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Cancer-associated Fibroblasts in Brain Metastases

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"*O binómio de Newton é tão belo como a Vénus de Milo.*

O que há é pouca gente para dar por isso."

Álvaro de Campos

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Resumo

As doenças oncológicas estão em vias de ser primeira causa de morte em países desenvolvidos. As metástases são responsáveis por cerca de 90% das mortes relacionadas com o cancro. A metastização é um processo complexo, que inclui invasão local, intravasação, circulação e sobrevivência, detenção num órgão distante, extravasação, micrometastização e colonização, no qual as células neoplásicas de um tumor primário disseminam para um órgão anatomicamente distante, adaptando-se ao novo microambiente.

Em particular, as metástases cerebrais têm geralmente mau prognóstico, com diminuição da qualidade de vida e curta sobrevivência. A sua incidência está a aumentar devido à melhoria no controlo terapêutico dos tumores primários, à evolução dos exames imagiológicos e ao aumento da incidência dos casos de melanoma. As atuais estratégias terapêuticas para metástases cerebrais, que são agressivas e têm uma eficácia limitada, incluem resseção tumoral, radioterapia holocraneana, radiocirúrgia e quimioterapia.

Tal como nos tumores extra-craneanos, o estroma tumoral tem um papel importante nesta patologia, uma vez que, fisiologicamente, o estroma cerebral é diferente do dos restantes órgãos, com células altamente especializadas.

Os fibroblastos associados a cancro (CAFs) são células fusiformes com núcleos alongados, para os quais ainda não existe um biomarcador específico, uma vez que constituem uma população celular heterogénea, com diversas origens e diversos mecanismos de ativação. Uma vez desencadeados estes mecanismos, os CAFs têm um papel determinante na progressão tumoral. Não só servem de suporte biomecânico, como produzem fatores que condicionam o comportamento tumoral, nomeadamente proliferação, transição epitélio-mesenquimatosa das células neoplásicas, migração e invasão, bem como remodelação da matriz extracelular, inflamação crónica dos tecidos, evasão ao sistema imune, angiogénese, resistência à terapêutica e formação do nicho pré-metastático.

À medida que as terapêuticas dirigidas ao estroma tumoral são exploradas e aplicadas com sucesso em tumores extra-craneanos, associado à pouca eficácia das terapêuticas atuais nas metástases cerebrais, cujos pacientes têm uma taxa de sobrevivência de

2,4% aos cinco anos após o diagnóstico, os CAFs surgem como um potencial e promissor alvo terapêutico.

Uma vez que fisiologicamente não existem fibroblastos no cérebro, mas tendo em conta que têm um papel determinante da progressão tumoral, o objetivo deste estudo é identificar fibroblastos em 138 amostras fixadas em formol e impregnadas em parafina de metástases cerebrais de tumores da mama, cólon, pulmão, rim e melanoma, os tumores que mais frequentemente metastizam para o cérebro, usando um painel de sete marcadores imunohistoquímicos [Colagénios I, IV, VI e VII, anti-fibroblasto TE-7, α-actina do músculo liso (SMA) e CD34]. Como controlos foram usados 30 casos de glioblastoma, tumor cerebral primário, e 22 casos de parênquima cerebral considerado normal.

De acordo com critérios de inclusão pré-definidos, foram observadas lâminas de arquivo de hematoxilina-eosina para seleção dos campos de interesse, com células tumorais e células do estroma morfologicamente compatíveis com fibroblastos. Foram criados 32 blocos de *tissue micro array*, com três cores por caso de metástase cerebral, um máximo de dezassete *cores* por bloco. Os blocos foram seccionados, tendo-se realizado a coloração de hematoxilina-eosina e as técnicas de imunohistoquímica em cortes de 3µm. As lâminas obtidas foram observadas por dois observadores independentes, especializados em neuropatologia. A marcação castanha de áreas do estroma foi considerada como marcação positiva, sendo posteriormente avaliada, segundo a sua intensidade, em fraca ou forte. As amostras foram microfotografadas e as áreas positivas foram medidas usando o *software* ImageJ. Considerando as áreas obtidas nas amostras dos controlos normais como um valor basal, este foi subtraído às áreas de marcação obtidas nas amostras tumorais, tanto das metástases como do tumor primário do cérebro. Foi realizada estatística descritiva usando o IBM SPSS e os gráficos foram feitos usando o SigmaPlot 12.0.

Os resultados obtidos suportam a presença de CAFs nas metástases cerebrais estudadas, apesar de nem todos os anticorpos usados serem específicos para fibroblastos. Todas as lâminas de hematoxilina-eosina obtidas mostraram estruturas e células compatíveis com a existência de CAFs na amostragem analisada. A maioria dos marcadores usados foram extensamente positivos para a quase totalidade das amostras estudadas, com 95,5% de positividade para o Colagénio I, 84,3% para o

Colagénio IV, 80,5% para o Colagénio VI, 75,8% para o anti-fibroblasto TE-7, 99,1% para a SMA e 100,0% para o CD34. O Colagénio VII marcou apenas 3,4% das amostras, pelo que não forneceu informações pertinentes à questão em estudo.

Os anticorpos Colagénio I, SMA e CD34 apresentam uma imunorreatividade fortemente positiva na maioria das amostras, enquanto que o Colagénio IV e Colagénio VI, bem como o anti-fibroblastos TE-7, foram fracamente positivos.

O Colagénio I foi o anticorpo com maior área de marcação, seguido do Colagénio IV, Colagénio VI e anti-fibroblastos TE-7. A SMA e o CD34 apresentaram menores áreas de marcação, comparativamente aos valores basais dos controlos normais.

O Colagénio I é o colagénio mais abundante no corpo humano e putativamente sintetizado pelos fibroblastos. Uma vez que os tumores atuam como feridas que nunca cicatrizam e que o Colagénio I é deveras importante nos processos de cicatrização, seria expectável que fosse positivo na maioria das amostras estudadas, o que foi confirmado pelos dados obtidos.

O Colagénio IV marcou membranas basais dos vasos sanguíneos, embora também se tenha observado áreas difusas fracamente marcadas, que podem corresponder a áreas de degradação de Colagénio IV ou áreas de membranas basais atípicas devido ao processo anormal de angiogénese, já sugerido como importante nos processos de metastização.

O Colagénio VI está frequentemente desregulado em tumores, sendo responsável por suporte estrutural, angiogénese, apoptose, proliferação, fibrose e inflamação.

O anti-fibroblasto TE-7 tem sido estudado como potencial biomarcador de fibroblastos. No entanto, marcou apenas 75,8% das amostras estudadas, na sua maioria fracamente, o que pode refletir uma expressão diferencial e, possivelmente, mais específica que os outros marcadores utilizados. A sua marcação foi idêntica às obtidas com Colagénio I e Colagénio IV, embora menos extensa.

A SMA, como esperado, marcou células de músculo liso presentes dos vasos sanguíneos e células aparentemente benignas de aspeto morfologicamente compatível com fibroblastos.

O CD34 é um marcador de células endoteliais, e como estas células são uma das fontes de CAFs, pensou-se que seria útil na determinação da origem deste, sem contrapartida no nosso estudo.

A identificação da presença de fibroblastos em metástases cerebrais conseguida neste estudo é apoiada por resultados semelhantes obtidos por outros grupos de investigação, mas englobando um número de amostras e de anticorpos substancialmente superior.

O estudo teve algumas limitações, sendo a mais importante a ausência de um biomarcador específico para CAFs, embora parcialmente ultrapassado com a utilização de um painel alargado de anticorpos.

Mais estudos sobre este assunto devem ser realizados. Alguns deverão recorrer à utilização de outros marcadores imunohistoquímicos, como a proteína específica de fibroblastos (FSP) 1. Igualmente, a contagem de células morfologicamente compatíveis com fibroblastos e o estudo de marcadores ao longo da linha evolutiva dos tumores (tumor primário, tumor metastático extra-craneano e metástase cerebral) deverão constituir outras tantas linhas de investigação.

A identificação de fibroblastos em metástases cerebrais aumenta o conhecimento oncobiológico desta patologia. Uma vez que as terapêuticas atuais são limitadas e pouco eficazes, e que a terapêutica dirigida a componentes do estroma tem vindo a ser estudada e utilizada com sucesso em variados tumores primários e metastáticos, a presença de CAFs em metástases cerebrais revela um novo alvo terapêutico, que pode revolucionar os atuais *standards* terapêuticos.

Palavras-chave

Anticorpo;

Estroma tumoral;

Fibroblastos associados a cancro;

Metástases cerebrais.

Abstract

Metastases are responsible for about 90% of cancer deaths and a major problem in cancer management. In particularly, the incidence of brain metastases, which are often a sign of poor prognosis, diminished quality of life and short survival, are increasing due to the better control of primary tumours, the evolution of imaging techniques and the incidence rise of melanoma. Actual strategies include whole brain radiotherapy, stereotactic surgery, surgical resection and chemotherapy, which are very aggressive and with limited effectiveness for the patient .Tumour stroma is a key player that can determine overall outcome in cancers throughout the body. Indeed, cancer-associated fibroblasts (CAFs) have a complex and critical role in cancer progression, and there is still no specific biomarker for them. The main goal of this research project is to identify fibroblasts in tissue stroma of 138 formalin-fixed paraffin embedded samples of the most common brain metastases (lung, breast, colon, kidney and melanoma), using an extended panel of the following immunohistochemical markers: Collagen I, Collagen IV, Collagen VI, Collagen VII, anti-fibroblast TE-7, α-Smooth muscle actin and CD34. Our data provides compelling evidence for the presence of CAFs in most frequent brain metastases, as our findings are similar to previous published studies. A better understanding of the crosstalk between cancer cells and CAFs can lead to the development of novel therapeutic agents and strategies. The identification of these cells increases the oncobiological knowledge of brain metastases and, as stroma-targeted therapies are being exploited and becoming more popular in primary tumours and extra-cranial metastasis, its identification can arise new and useful strategies in brain metastases management.

Keywords

Brain metastases;

Cancer-associated fibroblasts;

Marker;

Tumour stroma.

Abbreviations

α-SMA / SMA – α-smooth muscle actin;

BBB – Blood-brain barrier;

BCR/ABL – Breakpoint cluster region protein/Abelson tyrosine-protein kinase 1;

BMDC – Bone-marrow derived cells;

CAFs – Cancer-associated fibroblasts;

CNS – Central nervous system;

ColI – Collagen I;

ColIV – Collagen IV;

ColVI – Collagen VI;

ColVII – Collagen VII;

CTCs – Circulating tumor cells;

DAB – 3,3'-diaminobenzidine;

ECF – Extracellular fluid;

ECM – Extracellular matrix;

EGFR – Epidermal growth factor receptor;

EMT – Epithelial-mesenchymal transition;

EndMT – Endothelial-mesenchymal transition;

FFPE – Formalin-fixed paraffin-embedded;

FSP1 – Fibroblast-specific protein 1;

h – hour;

H&E – Hematoxylin-eosin;

HIF-1 – Hypoxia inducible factor-1;

MMP(s) – Matrix metalloproteinase(s);

ON – Overnight;

PBS – Phosphate buffer saline;

PTEN – Phosphatase and tensin homolog;

RT – Room temperature;

SDF-1 – Stromal cell-derived factor 1;

TE7 – Anti-fibroblast TE-7;

TGF – Transforming growth factor;

TMA(s) – Tissue micro array(s);

VEGF(R) – Vascular endothelial growth factor (receptor);

WHO – World Health Organisation.

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1. Introduction

Cancer is becoming the biggest cause of death in developed countries, mostly due to the improvements in healthcare concerning heart and vascular diseases. According to the World Health Organisation (WHO), in 2012, 8.2 million people died from cancer and 32.6 million people were living with cancer within a five-year diagnosis (1).

As a complex study subject, cancer cells are the core of many research projects. Furthermore, recent advances in this field suggest that both tumour microenvironment and stromal cells have an active and important role in tumorigenesis, cancer progression and disease outcome.

1.1. Metastases

Metastases are responsible for about 90% of cancer deaths and a major problem in cancer management. Metastization is a process in which cancer cells from a primary tumour disseminate to an anatomically distant organ and adapt to the new microenvironment (2). Yet, only a few are capable of completing the invasion-metastasis cascade (Figure 1.1).

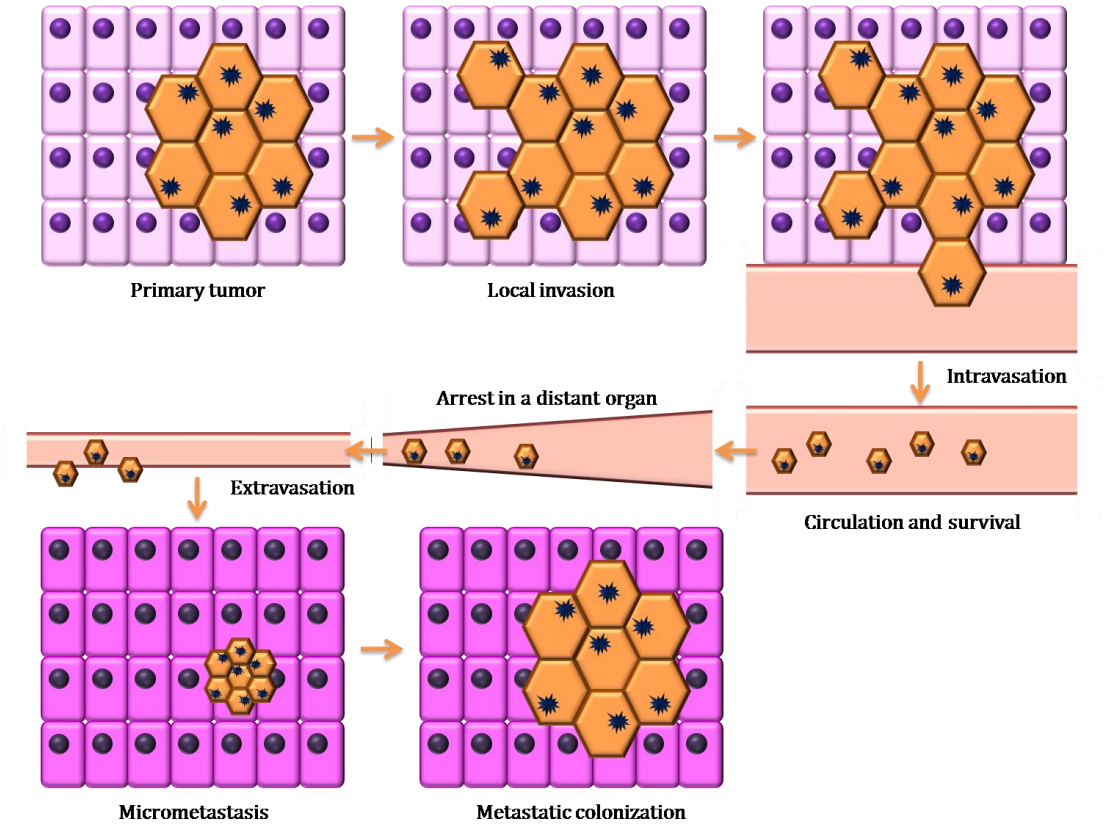

Figure 1.1. Invasion-metastasis cascade (inedited figure).

This complex multistage process of biological events is reviewed and discussed in several articles.

Firstly, cancer cells invade locally surrounding tissues on the primary site, with breach of the basement membrane (2). To accomplish it, epithelial-mesenchymal transition (EMT; Figure 1.2) promotes the ability of cancer cells to emigrate from the tumour. This process often requires signalling between cancer cells and neighbouring stromal cells, for example via transforming growth factor (TGF) β (3,4). It implies loss of polarity; shedding of cell-cell and cell-matrix interactions, with suppression of epithelial markers, like E-cadherin, and upregulation of mesenchymal markers in cancer cells, such as Vimentin; and acquisition of plasticity, contributing for the metastatic behaviour of cancer cells (4–6). Ultimately, loss of basement membrane allows a direct contact between cancer cells and tissue stroma.

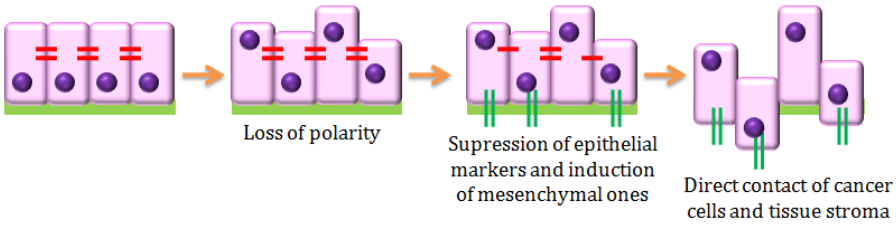

Figure 1.2. EMT. Adapted from Micalizzi DS, *el al* (6).

As the tumour progresses, stroma becomes more reactive, as in a healing wound or in chronic inflammation, and this set of cancer-stromal cells sustain some of the well-known hallmarks of cancer (Figure 1.3): evasion of growth suppressors; avoid immune destruction; replicative immortality; invasion and metastasis activation; angiogenesis induction; cell death resistance; cellular energetic deregulation and sustained proliferative signalling (7). These hallmarks correspond to several distinctive and complementary biological capabilities acquired during tumorigenesis and cancer progression.

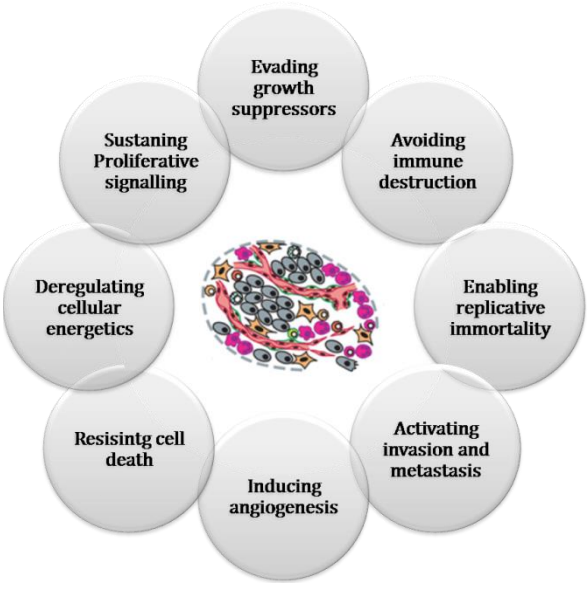

Figure 1.3. Hallmarks of Cancer. Adapted from Hanahan D, *et al* (7).

Cancer stroma, constituted by fibroblasts, myofibroblasts, endothelial cells, adipocytes and bone-marrow derived cells (BMDC), can enhance the aggressive behaviour of cancer cells, through the expression and/or secretion of key molecules. Examples of these are IL-6, Wnt5α and BMP4 expression by bone marrow-derived cancer-associated fibroblasts (CAFs) in gastric cancer (8), stromal cell-derived factor (SDF)-1 secreted by stromal fibroblasts in breast cancer (9) and IL-6 secreted by adipocytes in breast cancer (10).

The subsequent step is intravasation – the invasion of blood and lymphatic microvasculature by tumoral cells. Hematogenic pathway is the major mechanism of dissemination, where molecular changes allow tumoral cells to cross the barrier formed by pericytes and endothelial cells. Giampieri *et al* (11) have shown that constitutive TGFβ signalling promote single cell motility and intravasation, whether its inhibition had an opposite effect. Tumour cells are also capable of stimulating neoangiogenesis, *via* vascular-endothelial growth factor (VEGF) and fibroblast growth factor (7). Angiogenesis is a process in which new vessels are formed from existing ones. In contrast to normal blood vessels, the new ones are tortuous, leaky and instable(2,7), which causes hypoxia, a major player in tumour aggressiveness. In response to low oxygen tension, hypoxia inducible factor-1 (HIF-1) is stabilized, activating genes involved in angiogenesis (12), cell survival and therapy resistance (13), as well as organ-tropism (14).

Once inside the vessels, cancer cells have to be able to survive in the bloodstream. Circulating tumour cells (CTCs) have to overcome the potential damage by hemodynamic shear forces present in bloodstream and predation by innate immune cells, achieved through interactions with blood platelets (2). Additionally, CTCs are *anoikis*-resistant, as they have lost their normal anchorage to tissues, as in Notch signalling in prostate cancer, trough NF-κB activity increase (15); and Caveolin-1 upregulation in lung cancer, due to nitric oxide present in the microenvironment (16).

Both tissue anatomical configuration and vasculature layout allow a passive homing and extravasation of CTCs in certain organs. However, this is not enough to explain all observed metastases. There is a preferential pattern of target organs for certain tumours, supported by Page's "seed and soil" hypothesis (17). Some metastases are explained by an organ-specific tropism, orientated by specific genetic programs that

can overcome some of the incompatibilities between cancer cells and the new microenvironment. Various research groups have developed and tested this theory, in order to identify the involved genes, molecules and/or mechanisms. For instance, Bos *et al* (18) have identified three genes, cyclooxygenase COX2, Epidermal Growth Factor Receptor (EGFR) ligand HBEGF and α2,6-sialyltrnasferase ST6GALNAC5, that mediate breast cancer metastases to the brain. Likewise, Muller *et al* (19) were able to show the role of chemokines and their receptors in metastatic destination of CTCs in breast cancer. Finally, Minn *et al* (20) and Kang *et al* (21) have identified genes that also favour breast cancer metastases to lung, like CXCL1, HER2, matrix metalloproteinase (MMP) 1 and COX2, and to bone, like CXCR4, osteopontin, MMP-1 and IL-11, respectively.

A foreign microenvironment, with different stromal cells, extracellular matrix (ECM) components, available growth factors, cytokines and even microarchitecture, means that some form of adaptation must have to occur. Some primary tumours can produce factors capable of perturbing these new microenvironments, so that colonization is successful. Psaila and Lyden (22) reviewed the establishment of this "pre-metastatic niche", involving mobilization of BMDC into target organs to make the new environment more favourable to metastatic cells. These BMDC are VEGF receptor 1 (VEGFR-1) positive and appear to reach the pre-metastatic sites prior to CTCs (23).

The following and final step includes mesenchymal-epithelial transition of cells and colonization, which corresponds to the outgrowth of secondary lesions in the new organ, the most common ones being lung, liver, bone and brain. The endpoint is the formation of large macroscopic and clinically detectable metastases.

Management of metastatic disease is challenging, and most therapeutic strategies at this stage are based on slowing tumour growth or reducing metastatic tumour's side effects. Drug resistance is frequent for a great proportion of these patients, meaning that current strategies have a limited activity, as metastatic disease requires systemic therapeutic (2,24). Drug resistance can be inherited or acquired, trough the induction of drug transporters, activation of DNA repair, biotransformation of administered therapeutics, alterations in target proteins, changes in survival and/or apoptotic pathways or even due to intra-tumoral heterogeneity (24).

Tumours are known to have genetically distinct subpopulations of cancer cells, like Gerlinger *et al* have been exploring in renal carcinoma (25) and Navin *et al* in breast carcinoma (26), which supports the hypothesis that cancer can be described as an evolutionary model (24), with important implications to patients' management and outcome. Consequently, these subpopulations have different sensitivities to chemotherapy, different abilities to initiate tumours and different abilities to interact with other cells (24). For instance, Imatinib resistance in chronic myeloid leukaemia is associated with cancer cells that harbour Breakpoint Cluster Region protein/Abelson tyrosine-protein kinase 1 (BCR/ABL) kinase domain mutations (27,28). EMT is also a source of drug resistance. Thomson *et al* (29) correlated changes in epithelial and mesenchymal cell markers with sensitivity of non-small cell lung carcinoma cell lines to Erlotinib, an EGFR inhibitor, where the mesenchymal phenotype was linked to a poorer prognosis. Moreover, tumour microenvironment can influence drug resistance (24). Recent therapeutic guidelines consist in tumour microenvironment-targeted therapies, alone or in combination with systemic treatment, not only for primary tumours, to avoid or slow its progression down, but also for metastatic disease, to achieve a better outcome for the patient. For instance, advanced breast cancer has a high rate of bone metastases and the usage of biphosphonates not only improves the quality of life of advanced breast cancer patients, by acting on bone symptoms (30), but also reduces the incidence of bone metastases, due to inhibition of breast carcinoma cell adhesion to bone and tumour cell invasion (31,32). Bevacizumab, an angiogenic inhibitor, is another example, because, used in combination with chemotherapy, has effective results in managing advanced non-squamous non-small cell lung cancer (33).

1.2. Brain

The brain is one of the most common sites of secondary relapse.

In order to better understand brain metastases, it is important to recognize both normal and pathologic cellular variations.

Histologically, the brain has two major embryological cell lineages: cells of neuroectodermal origin, that include neurons, astrocytes, oligodendrocytes and ependymocytes; and cells of mesenchymal origin, that constitute specific structures like meninges, blood vessels and microglia (34).

Neurons (Figure 1.4, with black dashed arrow) are characterized by wide variations in size and shape and form a network of highly specific interconnections to collect and process information from sensory receptors to provide memory capacity and motor response. Morphologically, neurons are formed by a cell body, with a prominent round nucleus and nucleolus; an axon, that may extend for many centimetres and is responsible for transport, conduction of waves of depolarization and synaptic transmission; and numerous dendrites arising from the cell body, which connect through specialized cell junctions, designated synapses, with axons of other neurons (34,35).

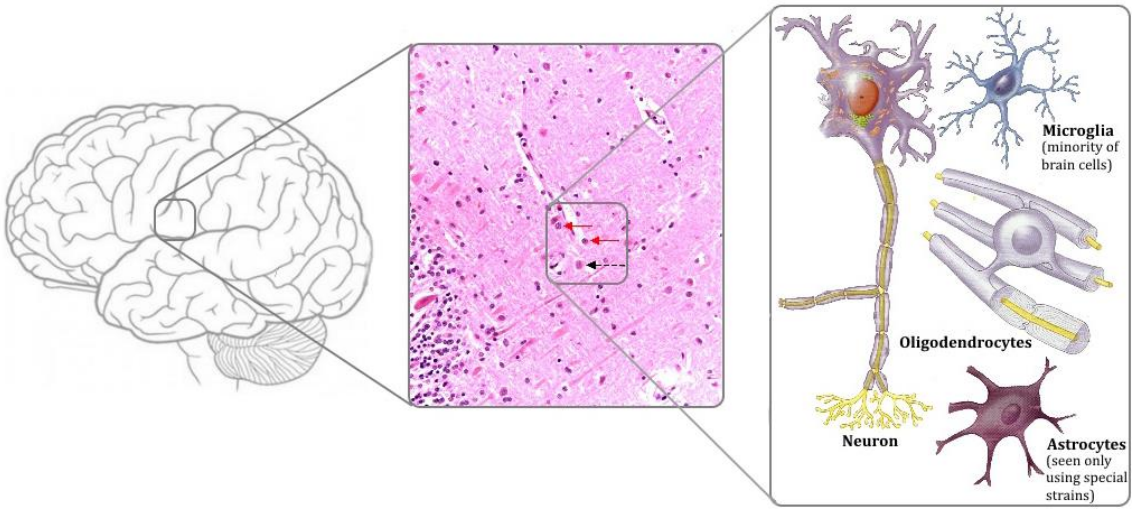

Figure 1.4. Histological and schematic representation of the main brain cells. Adapted from Tate P *et al* (36).

Astrocytes (Figure 1.4) are large cells with several roles within the central nervous system (CNS), including maintenance of integrity of the blood-brain barrier (BBB), structural scaffold, regulation of exchange of fluid, metabolites and ions between extracellular fluid (ECF) and blood, and absorption of excessive potassium ions and neurotransmitters from the ECF around axons and synapses. Upon damage of the CNS, astrocytes proliferate to infill affected areas. To fulfil their roles, astrocytes have cytoplasmic extensions that touch neurons, only observable by using special stains (34,35).

Oligodendrocytes (Figure 1.4, with red full arrow) are responsible for formation and maintenance of CNS myelin sheaths, important for isolation and adjustment of electrical conduction in axons, and are frequently found immediately adjacent to neuronal cell bodies (34,35,37).

Microglia (Figure 1.4) are specialized macrophages that become active in response to CNS injury or disease (34,35).

1.2.1. Brain Metastases

Brain metastases are often a sign of poor prognosis, diminished quality of life and short survival. Langley and Fidler (38,39) have two recent reviews on this theme. Patients may develop neurological deterioration due to destruction and/or displacement of normal brain parenchyma by the tumour, as well as by peritumoral edema, increased intracranial pressure and/or vascular compromise (38). Most common primary tumours with brain metastases include breast, colon, lung and kidney cancer as well as melanoma (Figure 1.5). The incidence of brain metastases is increasing due to a better control of primary tumours, the evolution of imaging techniques and the incidence rise of melanoma (38,40). However, little is still known about the mechanisms involved in these processes.

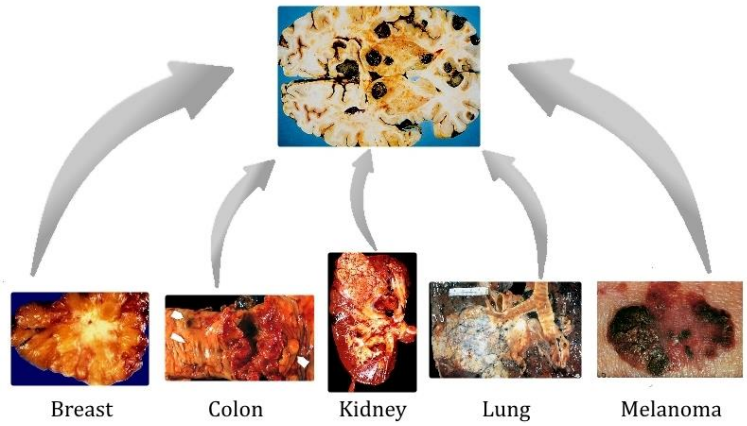

Figure 1.5. Most common primary tumours with brain metastases. Adapted from Kumar V *et al* (41)

BBB is responsible for a tight regulation of vascular access to the brain. It is composed by a layer of endothelial cells and astrocytes, representing an obstacle to reach brain parenchyma (39). Infiltration of CTCs into the brain requires disruption of the BBB and metastatic cells with highly , yet not fully characterized, specialized functions (42).

Stroma also plays a particularly important role in brain metastases, as brain parenchyma is very different from other organs, with highly specific cells. Angiogenesis-dependence varies according to the primary tumour, however most metastases promote vascular remodelling through splitting of existing dilated blood vessels, or sprouting from pre-existent ones. Normal endothelial cells produce

molecules to restrict the movement of toxic substances into the brain, rendering access of several systemic therapies (38,39).

Therapeutic approaches to brain metastases are therefore difficult, limited and include whole brain radiotherapy, stereotactic surgery, surgical resection and chemotherapy. However, the effectiveness of these options depend on patient's characteristics, such as age, functional status, primary tumour, extension and number of brain lesions and prior therapies (43). It is urgent to develop new strategies concerning the management of brain metastases, as median survival in these patients is very low, with a survival rate middling 2,4% at five years after diagnosis (43). Stroma-targeted therapy may improve already known treatments, and it is already performed in extra-cranial metastases (30–33).

1.3. Cancer-associated Fibroblasts

As discussed above, stroma is a key player that can determine overall outcome in cancers throughout the body, such as colorectal cancer (44). Cancer cells rely on a complex microenvironment of ECM along with several types of stromal cells, namely fibroblasts (or CAFs), endothelial cells and immune cells.

CAFs, like their homonymous normal cells, are large spindle-shaped cells, with elongated nuclei. As there are no specific biomarkers for CAFs, several putative markers have been studied, so that the characterization of these cells is made by using a combination of different immunhistochemical markers, namely α-smooth muscle actin (α-SMA), desmin, vimentin, CD14, CD31, CD34, and several collagens, among others (45–49).

The determination of specific biomarkers for CAFs is difficult because they are a heterogeneous population with multiple origins (Figure 1.6). Generally, local fibroblasts and myofibroblasts are considered the most obvious origin of CAFs(50). However, they can also arise from epithelial cancer cells through EMT(51), BMDC (52,53), adipose tissue-derived stem cells (53) and endothelial cells, through endothelial-mesenchymal transition (EndMT) (54).

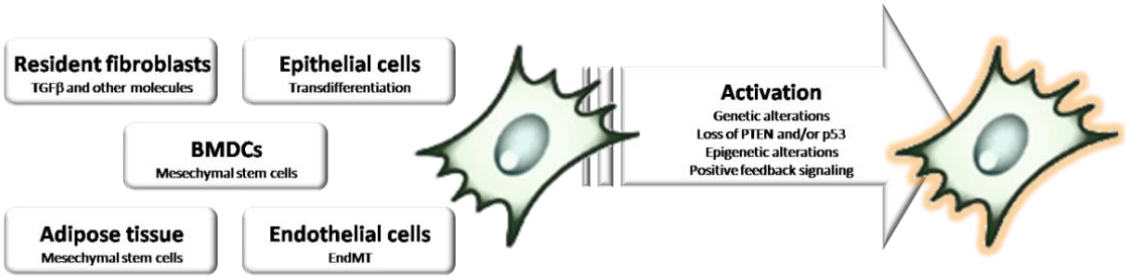

Figure 1.6. CAFs: sources and hypothesis of activation (inedited figure).

Orimo *et al* (9) demonstrated that, besides being biologically different, CAFs are more competent than normal fibroblasts, as they can maintain and sustain their activated phenotype. The underlying processes are not clear, but several research groups raised some hypothesis (Figure 1.5). These include genetic alterations (55), loss of phosphatase and tensin homolog (PTEN) and/or p53 with clonal selection of the most favourable phenotype (56), epigenetic alterations (57) or even positive feedback signalling created by autocrine loops, like TGFβ alone or in combination with SDF-1 autocrine signalling, which is initially triggered by cancer cells (50). TGFβ is a molecule with a dual function: while in early stages of carcinogenesis, it may function as a tumour suppressor (58), in advanced stages it promotes cancer progression and metastases (59).

Once activated, CAFs have a complex and critical role in cancer progression (Figure 1.7).

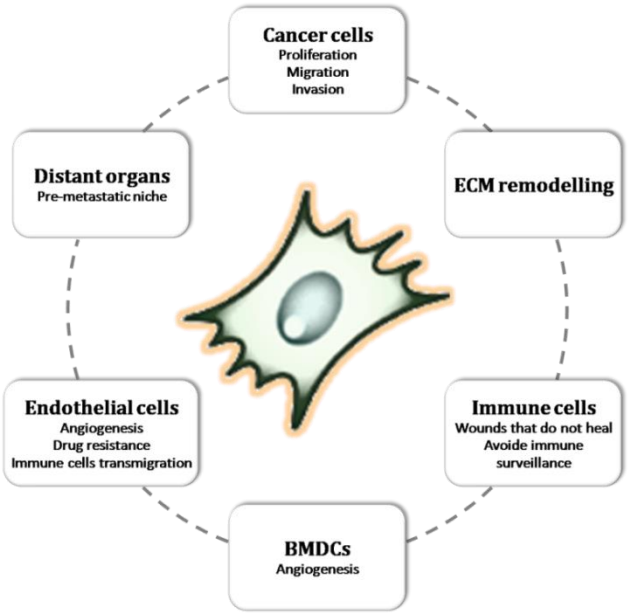

Figure 1.7. Role of CAFs in cancer and its relation with the hallmarks of cancer (inedited figure).

Not only do CAFs respond to cancer cells' stimuli and provide biomechanical support as part of tumour stroma, but they are also a source of different factors, such as cytokines, chemokines and growth factors, that can modulate cancer behaviour (9,60,61). Direct stimulation of cancer cells by CAFs promotes, for instance, hepatic and endometrial cancer cell proliferation (9,62–64), EMT of breast and prostate cancer cells (60,65), migration and invasion (61,66).

In addition, CAFs have a major role in ECM remodelling, just like normal fibroblasts. In solid tumours, an increase of matrix deposition with progressive stiffening is commonly observed. This desmoplasia, not exclusive of primary tumours, has been associated with poor prognosis (23,67,68). Furthermore, CAFs produce MMPs responsible for proteolytic degradation, allowing migration of not only isolated but also clusters of cancer cells (9).

Another role of CAFs is the formation of the pre-metastatic niche (23,49), which facilitates homing and colonization of secondary organs by CTCs. Duda *et al* (49) studied this hypothesis in lung metastases. In the analysed samples, tumours were described to shed not only single cells but also clumps with an increased metastatic potential into the blood stream. Those clumps were composed by cancer cells and stromal cells expressing mostly α-SMA and fibroblast-specific protein 1 (FSP1). Moreover, depletion of CAFs resulted into a significant growth delay in primary tumours, as well as a decrease of metastatic nodules in mice, suggesting that these stromal cells play an important role in lung metastases (49). This study also put forward the possibility that CAFs are relevant to brain metastases from lung, breast, kidney and endometrial cancers, by using α-SMA and CD10. These two markers showed positivity in benign-appearing stromal cells with typical fibroblast morphology in brain metastases (49).

Chronic inflammation was first hypothesized as a contributor to tumorigenesis by Virchow (69). Furthermore, cancer is commonly referred to as "wounds that do not heal" (70), because inflammation allows cancer cells to stay in contact with existing growth factors in the ECM and to avoid immune surveillance. CAFs secrete, for example, CCL2, a chemoattractant for monocytes and other immune cells (71), and IL-6, COX-2 and CXCL1 in breast and ovarian cancer (72), which contribute to an immunessuppressive microenvironment and, ultimately, to immune evasion by cancer

cells. Another study (73) demonstrated that CAFs isolated from metastatic melanoma samples could interfere directly with natural killer (NK) cells, resulting in a reduced cytolytic activity, affecting, therefore, the ability of NK cells to kill melanoma cells.

Another well described function of activated CAFs is the regulation of angiogenesis, by secreting pro-angiogenic growth factors, like VEGF and SDF-1 (9,49). The resulting leaky vessels trigger a high interstitial fluid pressure in the tumour, creating a mechanical barrier to intra-tumoral therapeutic uptake and affecting the immune cell transmigration into the stroma. In addition, CAFs can recruit BMDCs that act as endothelial progenitors and are incorporated in the neovasculature (9).

In brain metastases, Choi and colleagues (74) have demonstrated that CAFs can induce BBB disruption due to the increase of permeability, and consequent transmigration of breast cancer cells.

CAFs have several important roles in tumour progression. A better understanding of the crosstalk between cancer cells and CAFs can lead to the development of novel therapeutic agents and strategies. As stromal-targeted therapy is being exploited with interesting results in several studies and clinical trials, CAFs appear as a promising therapeutic target, as they are more stable than cancer cells (62). The rationale of this approach, reviewed by Togo *et al* (75), is based on aiming tumour signals that activate CAFs or CAFs themselves, which can promote elimination of CAFs or their signals and interactions with cancer, immune and endothelial cells. This leads to attenuated tumour growth (9,64), decreased angiogenesis (9,64), improved anti-tumoral drug delivery (76) and even restored immune response (64,77). Yet, as in other therapeutic strategies, several problems can arise. As there are no specific biomarkers for CAFs, actual strategies can also aim for normal fibroblasts, present in non-tumoral tissues, with no knowledge of potential and possible extension of side effects. Another problem is related to chronic inflammation and fibrosis caused by chemotherapy, like in esophagic cancer (78), and surgical interventions. Inflammation is a hallmark of cancer, as described above, and if a treatment can induce it, therapeutic effectiveness is not accomplished. Indeed, more studies are needed in order to correctly determine the total effect of these strategies on cancer patients.

2. Goals

The number of patients with brain metastases is increasing, as advances in oncologic disease are made. There are very few published studies correlating CAFs with brain metastases as there are no fibroblasts in the brain parenchyma in physiologic conditions. Nevertheless, these stromal cells have many different and determinant roles in cancer progression. So, it is important to correctly identify CAFs in order to improve not only the knowledge of metastatic tumours, but also to increase the number of potential therapeutic targets, as tumoral stroma is an important component in cancer management.

The main goal of this research project is to identify fibroblasts in brain metastases stroma of different primary tumours, using an extended panel of immunohistochemical markers. This will allow the assessment of the following questions:

- Are there CAFs in the most frequent brain metastases (breast, colon, lung and kidney and melanoma)?
- Is there overlapping staining of the studied markers?
- Do these markers have a particular pattern depending on the primary tumour?
- Is there a relation between the different markers that could indicate a possible origin of CAFs?

3. Ethical Considerations

The samples used in the current research project were obtained in a diagnosis context and are part of the general sample archive of the *Laboratório de Neuropatologia*, *Hospital Santa Maria*, *Centro Hospitalar de Lisboa Norte*. Only samples with enough biological tissue were selected (see Table 4.1), in order to allow future case review or additional studies.

The patients' confidentiality was maintained as samples were identified by their registration number or, in a later phase, by their position in tissue micro arrays (TMA) blocks and not by the patients' name or any other personal identification. Collected data will not be disclosed under any circumstance.

This study was performed on formalin-fixed paraffin embedded (FFPE) material with a maximum of fifteen years old. At the time of collection, informed consent was not collected in *Laboratório de Neuropatologia* and, since these tumours are very aggressive, it is difficult, if not impossible, to get informed consents for these samples at the present.

The performed techniques did not invalidate the availability of archive material for each patient, in case it is needed. The use of TMAs ensured the preservation of tissues for further study, reducing the cost of the technique. Both TMA blocks created and slides obtained will be archived in the laboratory.

The project was approved by the Ethical Commission at 2014, October 29th, with the number 556/14.

4. Material and methods

4.1. Biological samples

In a preliminary phase of archive research, neuropathological diagnosis of every patient's case was confirmed in the reports, starting from the most recent to older ones. Samples were selected according to inclusion and exclusion criteria, as described below (Table 4.1).

Table 4.1. Inclusion and exclusion criteria for biological samples selection

Inclusion Criteria	Exclusion Criteria
Neuropathological diagnosis (glioblastoma and cancer metastases from breast, colon, lung, kidney and melanoma)	Different surgeries from the same patient (only the most recent one was used)
Sample age (starting from recent to older samples)	Aggressive procedures (samples with marked cauterization or decalcification artefacts were excluded)
Sample size (future cases review or additional studies were assured; small biopsies were avoided)	FFPE samples availability (those not available in the archive due to complementary studies were excluded)

According to the laboratory's casuistic and the criteria above, a total of one hundred and thirty-eight FFPE samples of brain metastases, from breast (thirty), kidney (twenty-eight), lung (thirty) and colorectal cancer (thirty) and melanoma (twenty), were selected. Apart from these, thirty samples of primary brain tumour and twenty normal brain samples were also selected, as controls. The primary brain tumour used was glioblastoma, the most aggressive astrocytic tumour, classified as grade IV, according to the WHO (79). The "normal" brain control belongs to non-tumoral areas from tumour samples, as distant from the tumour area as possible, as it is very difficult to have non-pathological brain in neuropathology.

Hematoxylin-eosin (H&E) slides from the selected cases were microscopically observed by three independent observers, in order to select fields with tumoral cells and stromal cells morphologically compatible with fibroblasts.

TMAs (Figure 5.1) were built with a maximum of seventeen cores, using a 3mm skin punch biopsy kit, in a total of thirty-two TMAs. Metastatic samples were collected in triplicate, whereas only one core was collected from control samples (80,81).

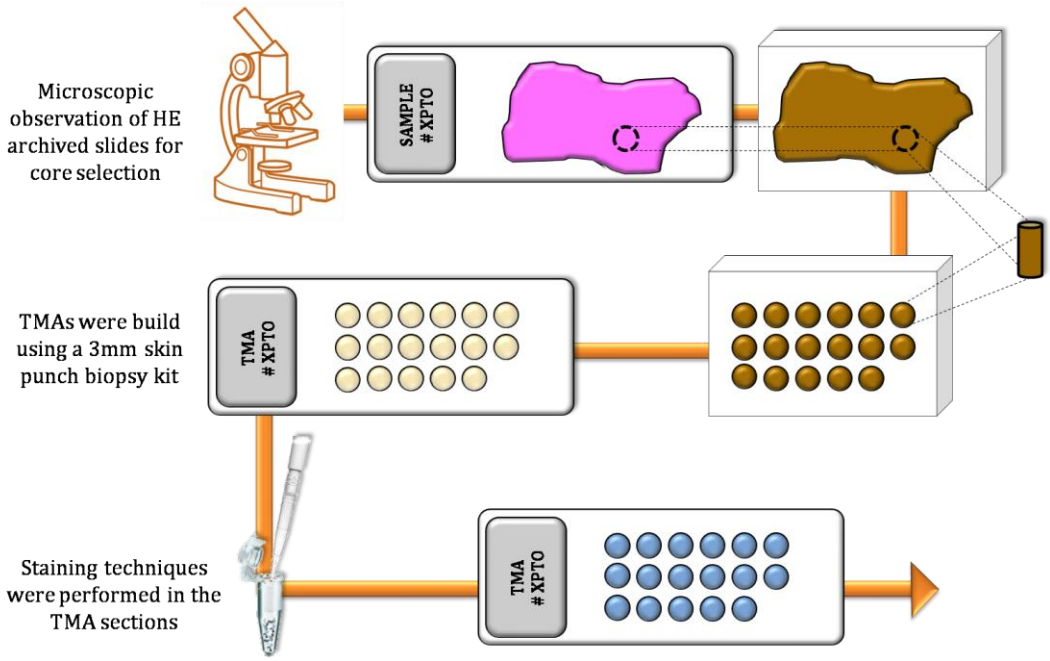

Figure 4.1. TMAs (inedited figure).

For the following techniques, 3µm sections were obtained, using a Minot microtome (Leica RM2145) and SuperFrost™ microscope slides, dried at 60°C for an hour and then cooled to room temperature.

4.2. Hematoxylin-Eosin Staining

H&E staining (appendix I) provides an overall morphologic review of the tissue, allowing the identification of several tissue structures and constituting the basis of contemporary cancer diagnosis. In this project, H&E staining was also used to confirm the correct sample collection for TMAs. Sections were dewaxed in xylene and rehydrated through graded alcohols to tap water. Afterwards, slides were immersed in hematoxylin for four minutes, washed in tap water, differentiated for two seconds and washed again in tap water to stop any chemical reactions. Sections were blued in 1% ammoniacal water for about fifteen seconds and washed with tap water. After staining with 1% alcoholic eosin for ten minutes, slides were dehydrated, cleared and mounted with synthetic mounting media, Entellan®.

4.3. Immunohistochemistry

Sections were dewaxed and rehydrated through graded alcohols to water, as in the previous procedure, for immunohistochemistry (appendix II). Endogenous peroxidase was blocked with a hydrogen peroxide solution for twenty to twenty-five minutes. Microwave (Samsung) antigen retrieval was performed, for ten minutes at 600 watts followed by twenty minutes at 850 watts. Slides were rinsed in phosphate buffered saline (PBS) and incubated with the following primary antibody: Collagens I (ColI), IV (ColIV), VI (ColVI) and VII (ColVII), anti-fibroblast TE-7 (TE7), α-SMA (designated from now on as "SMA") and CD34 (for additional details and working conditions see Table 4.2).

Table 4.2. Antibodies and respective conditions used in the present study

Antibody	Manufacturer Code Clone	Staining	Internal control	Conditions
Collagen I	Abcam ab34710 N/A	M	Connective tissue (82)	1:500 ON at 4°C
Collagen IV	Dako M 0785 CIV 22	E	Basement membranes (83)	1:100 1h at RT
Collagen VI	Leica NCL-COLL-VI 64C11	C	Microfibrillary structures localized close to cells, nerves and blood vessels (84)	1:50 1h at RT
Collagen VII	Leica NCL-COLL-VII LH7.2	M	Basal lamina of stratified epithelia such as epidermis, oral, esophageal, epithelium and urothelium of the bladder, as well as sweat gland epithelium or breast epithelium, among others (85)	1:50 1h at RT
Anti-Fibroblast TE-7	Millipore TE-7 TE-7	C	(Developed for the assessment of thymic epithelial culture contamination with fibroblasts) Used only in research (86)	1:25 ON at RT
SMA	Leica NCL-SMA ASM-1	C	Smooth muscle cells in blood vessel walls, gut wall, myometrium and arrectores pili of skin (87)	1:50 1h at RT
CD34	Leica NCL-End QBEnd/10	C	Endothelial cells (88)	1:100 1h at RT

Legend: N/A – Not applicable; C – Cytoplasm; E – Extracellular; M – Membrane; N – Nuclear; h – hour; RT – Room temperature; ON – overnight.

Sections were then rinsed and incubated with a secondary antibody, Dako REAL™ Envision™ HRP Rabbit/Mouse or Novocastra™ Novolink™ Polymer Detection System, depending on the primary antibody, for two hours at room temperature. After incubation, slides were rinsed, and stained with 3,3'-diaminobenzidine (DAB) solution for five minutes and rinsed in tap water. Slides were counterstained for four minutes with Harris Hematoxylin, differentiated, blued with 1% ammoniacal water, dehydrated, cleared and mounted with synthetic mounting media, Entellan®.

In order to minimize possible differences in immunostaining, the technique was performed under the same conditions (for further details, see appendix II).

4.4. Data Analysis

All slides were analysed by two independent, experts in neuropathology, observers using a bright-field microscope. Two different features were taken into account: (1) positivity, corresponding to brown staining in tumoral stroma; and (2) staining intensity, as weak or strong. Data from the first observations was subjected to a second evaluation by the observers, at the same time, due to minor disagreements in staining intensity and errors (like missed evaluation from some cores, during the first assessment).

Slides were microphotographed using the binocular microscope Leica DM 2000 LED, equipped with a digital camera Leica ICC 50HD and the Leica Application Suite LAS EZ (version 2.1.0) software. Images were acquired, using a 100x magnitude, in .tiff format, with a 16-bit of bit depth and a resolution of 2048x1536 dots per inch (dpi).

Positive staining areas were measured using ImageJ. Normal control positive areas, considered a basal value, were subtracted to the ones obtained in tumoral samples. Descriptive statistical analysis was performed using IBM SPSS and graphics were made using SigmaPlot 12.0.

5. Results

The sample used in the present project was composed by four hundred and sixty-six TMA cores containing thirty breast metastases, thirty colon metastases, twenty-eight kidney metastases, thirty lung metastases and twenty melanoma metastases (Table 5.1). As controls, it was used twenty samples of adjacent, but distant as possible, brain parenchyma considered normal found in the tumour samples themselves, designated as “normal”, and thirty glioblastomas as primary brain tumour. H&E staining and a panel of seven antibodies were performed in all samples, resulting in two hundred and fifty-six slides.

Table 5.1. Sample characterization by origin of the primary tumour (metastases samples collected in triplicate)

Samples	Origin	Number of Samples	Number of TMAs Cores	% of total
Metastasis	Breast	30	90	19.3%
Metastasis	Colon	30	90	19.3%
Metastasis	Kidney	28	84	18.0%
Metastasis	Lung	30	90	19.3%
Metastasis	Melanoma	20	60	12.9%
Control	Normal	22	22	4.7%
Control	Primary Brain	30	30	6.4%
	Total	190	466	100.0%

H&E staining showed morphologically structures compatible with the presence of fibroblasts in brain metastases, particularly spindle-shaped cells with elongated nucleus (Figure 5.1).

Although not all antibodies were positive in every sample slides (Table 5.2, Figure 5.2), like Collagen VII, with only 3.4% of positivity, the great majority of them stained positively for the remain immunohistochemical panel used: 95.5% for Collagen I; 84.3% for Collagen IV; 80.5% for Collagen VI; 75.8% for anti-fibroblast TE-7; 99.1% for SMA; and 100.0% for CD34.

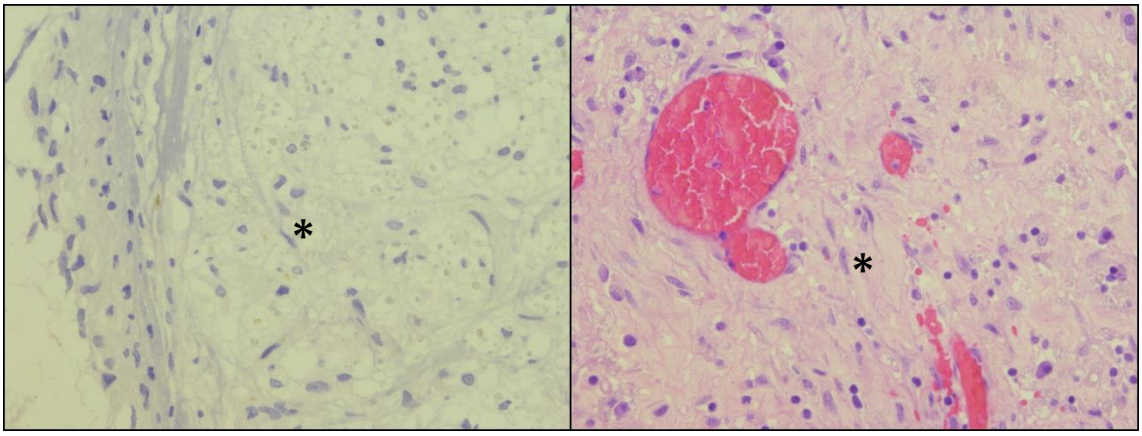

Figure 5.1. Example of H&E staining demonstrating structures morphologically compatible with the presence of fibroblasts (*) in brain metastases (hematoxylin on the left and H&E on the right).

Table 5.2. Immunohistochemical positive staining

Antibody		Positive	Negative	Total
ColI	*Frequency*	445	21	466
	% within Positivity	**95.5%**	4.5%	100.0%
ColIV	*Frequency*	393	73	466
	% within Positivity	**84.3%**	15.7%	100.0%
ColVI	*Frequency*	375	91	466
	% within Positivity	**80.5%**	19.5%	100.0%
ColVII	*Frequency*	16	450	466
	% within Positivity	**3.4%**	96.6%	100.0%
TE7	*Frequency*	353	113	466
	% within Positivity	**75.8%**	24.2%	100.0%
SMA	*Frequency*	462	4	466
	% within Positivity	**99.1%**	0.9%	100.0%
CD34	*Frequency*	466	0	466
	% within Positivity	**100.0%**	0.0%	100.0%

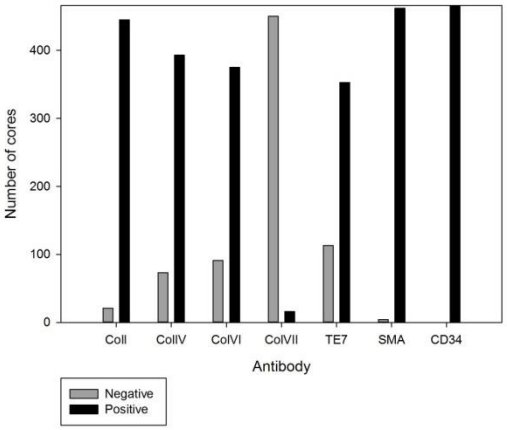

Figure 5.2. Immunohistochemical positive staining.

Analysing data according to the tumour origin (Figure 5.3), all metastatic samples showed 100.0% positivity for Collagen I, as well as for CD34. With the exception of melanoma (96.7%), SMA was also 100.0% positive in the remaining samples.

Breast samples showed 91.1% positivity for Collagen IV, 76.7% for Collagen VI and 94.4% for anti-fibroblast TE-7.

Colon samples showed 90.0% positivity for Collagen IV, 75.6% for Collagen VI and 83.3% for anti-fibroblast TE-7.

Kidney samples showed 71.4% positivity for Collagen IV, 82.1% for Collagen VI and 72.6% for anti-fibroblast TE-7.

Lung samples were 81.1% positive for Collagen IV, 90.0% for Collagen VI and 76.7% for anti-fibroblast TE-7, whereas only 3/90 (3.3%) cores were positive for Collagen VII.

Melanoma samples stained positively in 86.7% for Collagen IV, 96.7% for Collagen VI, 21.7% for Collagen VII and 96.7% for anti-fibroblast TE-7.

In normal control samples, 4.5% cores were positive for Collagen I, 90.9% for Collagen IV, 9.1% for Collagen VI, 22.7% for anti-fibroblast TE-7, 95.5% for SMA and 100.0% for CD34.

The primary brain control (Figure 5.3) stained 100.0% for Collagen I, 83.3% for Collagen IV, 93.3% for Collagen VI, 70.0% for anti-fibroblast TE-7, 96.7% for SMA and 100.0% for CD34.

Although neither of them is specific for fibroblasts, the used antibody panel, supported by reviewed literature, demonstrated cells and structures compatible with the presence of fibroblasts (Figure 5.4), with even some overlapping staining of some antibodies in a few samples (see lung sample in Figure 5.4).

Staining intensity, showed in Table 5.3 and Figure 5.5, was evaluated as “weak” and “strong” and is roughly related to antigen quantity. Collagen I, SMA and CD34 were strongly positive (93.1%, 95.5% and 97.0% respectively), whereas Collagens IV and VI and anti-fibroblast TE-7 had weak staining intensity in most metastatic cores. Both results were consistent when analysing by tumour origin, including not only metastatic, but also primary brain tumours (Figure 5.6). Collagen IV, besides the expected positive basement membranes staining, showed also diffuse weakly staining areas in some metastatic samples (Figure 5.7).

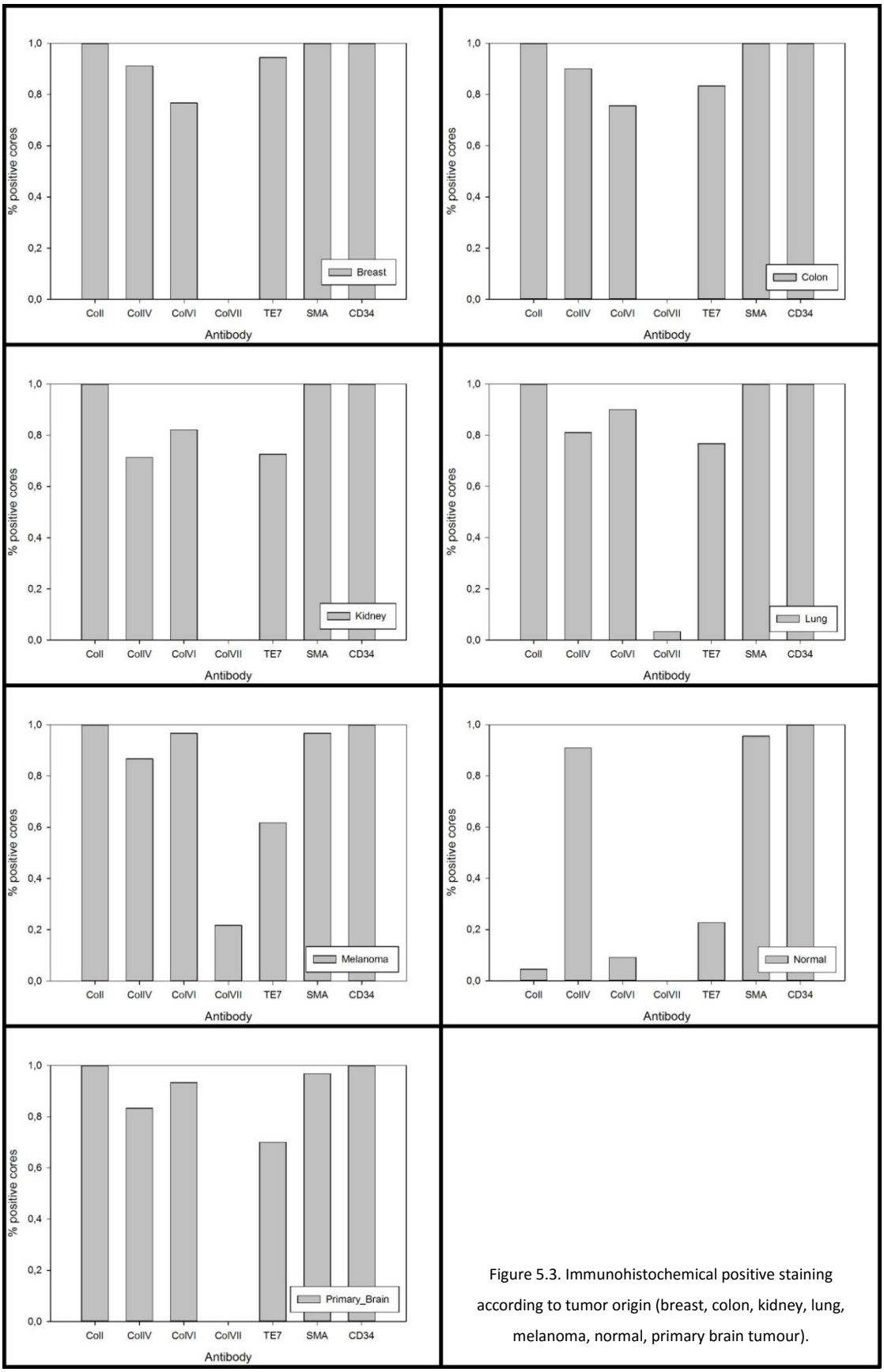

Figure 5.3. Immunohistochemical positive staining according to tumor origin (breast, colon, kidney, lung, melanoma, normal, primary brain tumour).

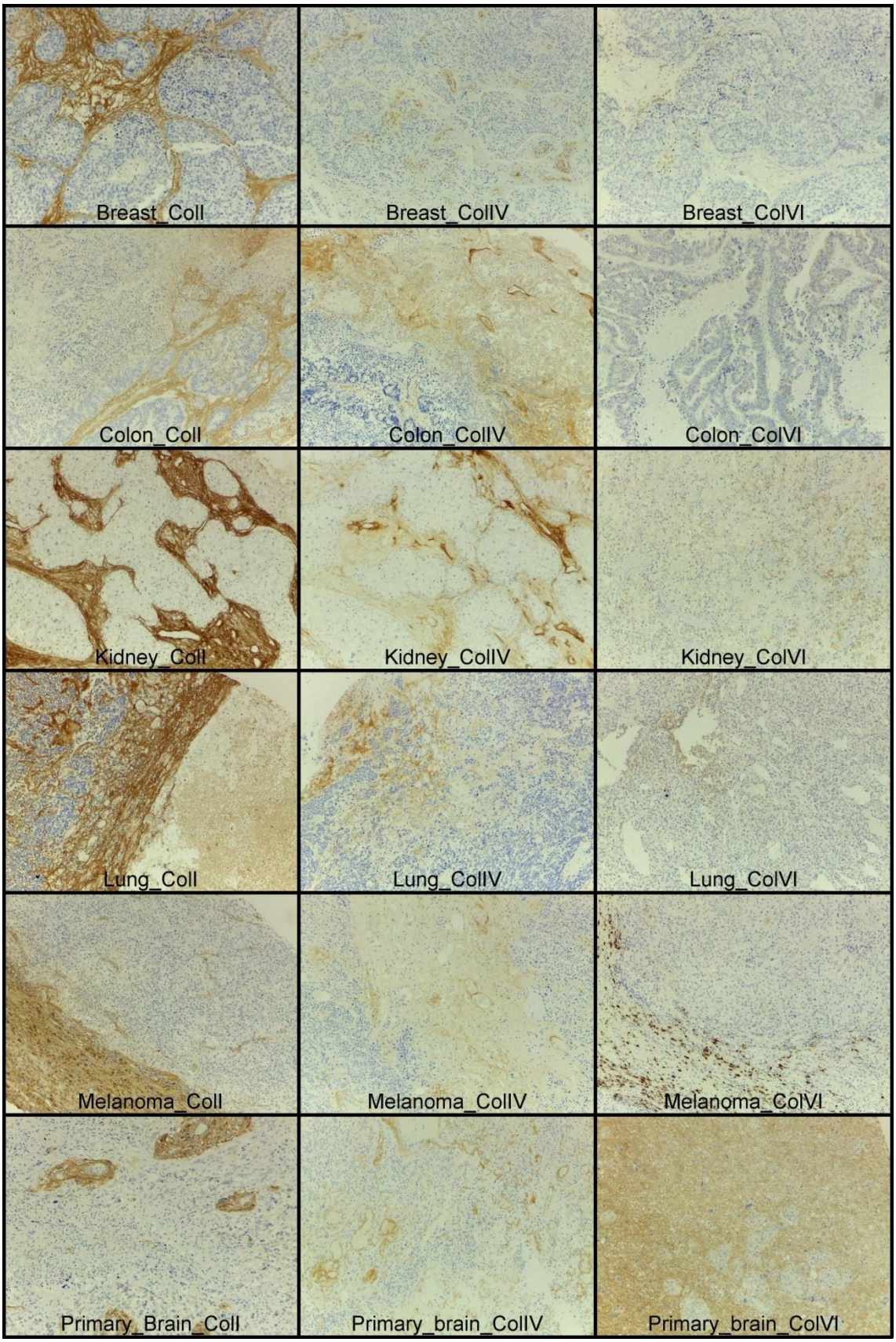

Figure 5.4. Immunohistochemical positive staining according to tumour origin (breast, colon, kidney, lung, melanoma, normal, primary brain tumour).

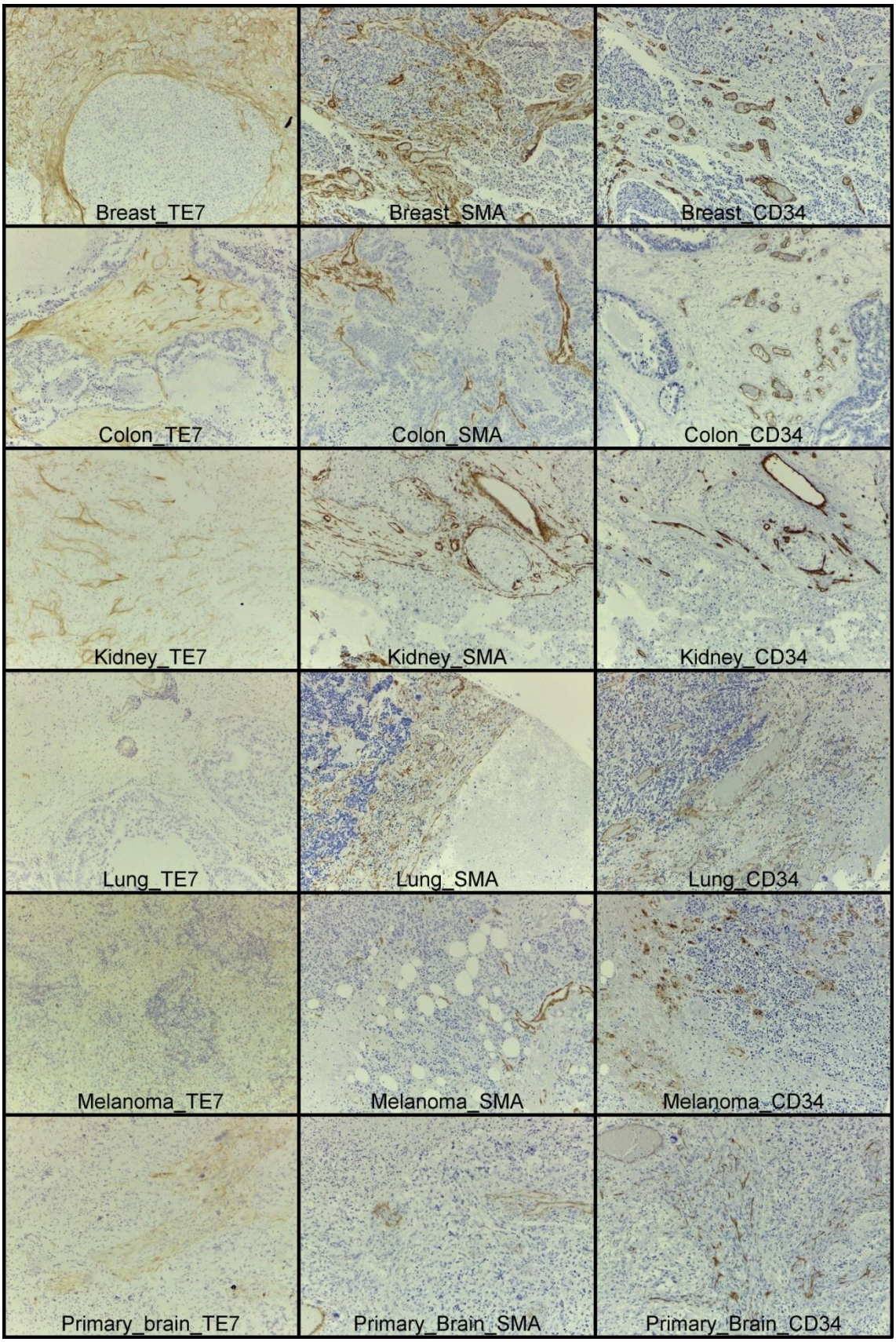

Figure 5.4. (continued).

Table 5.3. Immunohistochemical positive staining intensity, evaluated as “weak” and “strong”

Antibody		Negative	Positive | Weak	Positive | Strong
ColI	*Frequency*	21	11	**434**
	% within Positivity	4.5%	2.4%	**93.1%**
ColIV	*Frequency*	73	**341**	52
	% within Positivity	15.7%	**73.2%**	11.2%
ColVI	*Frequency*	91	**333**	42
	% within Positivity	19.5%	**71.5%**	9.0%
ColVII	*Frequency*	450	14	2
	% within Positivity	96.6%	3.0%	0.4%
TE7	*Frequency*	113	**274**	79
	% within Positivity	24.2%	**58.8%**	17.0%
SMA	*Frequency*	4	17	**445**
	% within Positivity	0.9%	3.6%	**95.5%**
CD34	*Frequency*	0	14	**452**
	% within Positivity	0.0%	3.0%	**97.0%**

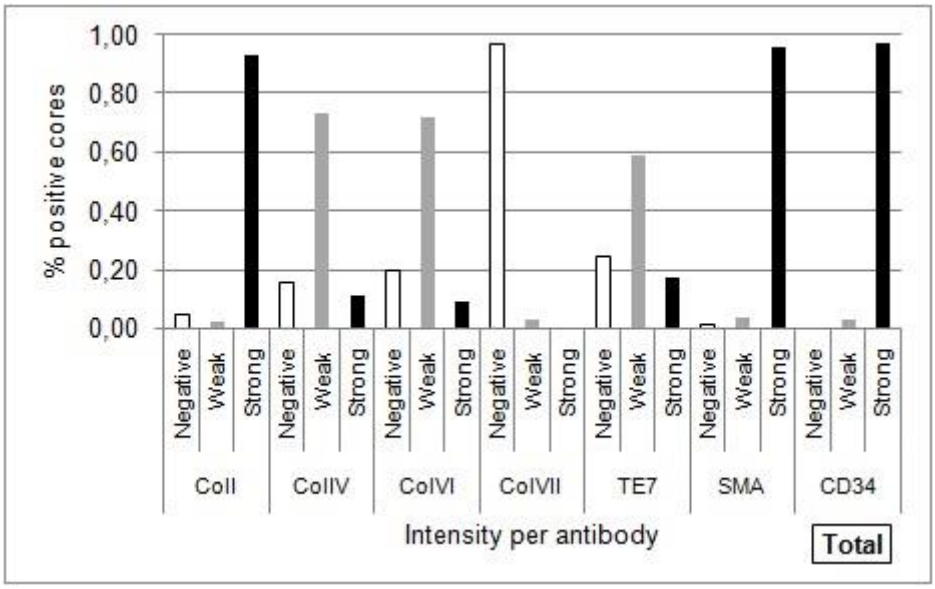

Figure 5.5. Immunohistochemical positive staining intensity, evaluated as “weak” and “strong”.

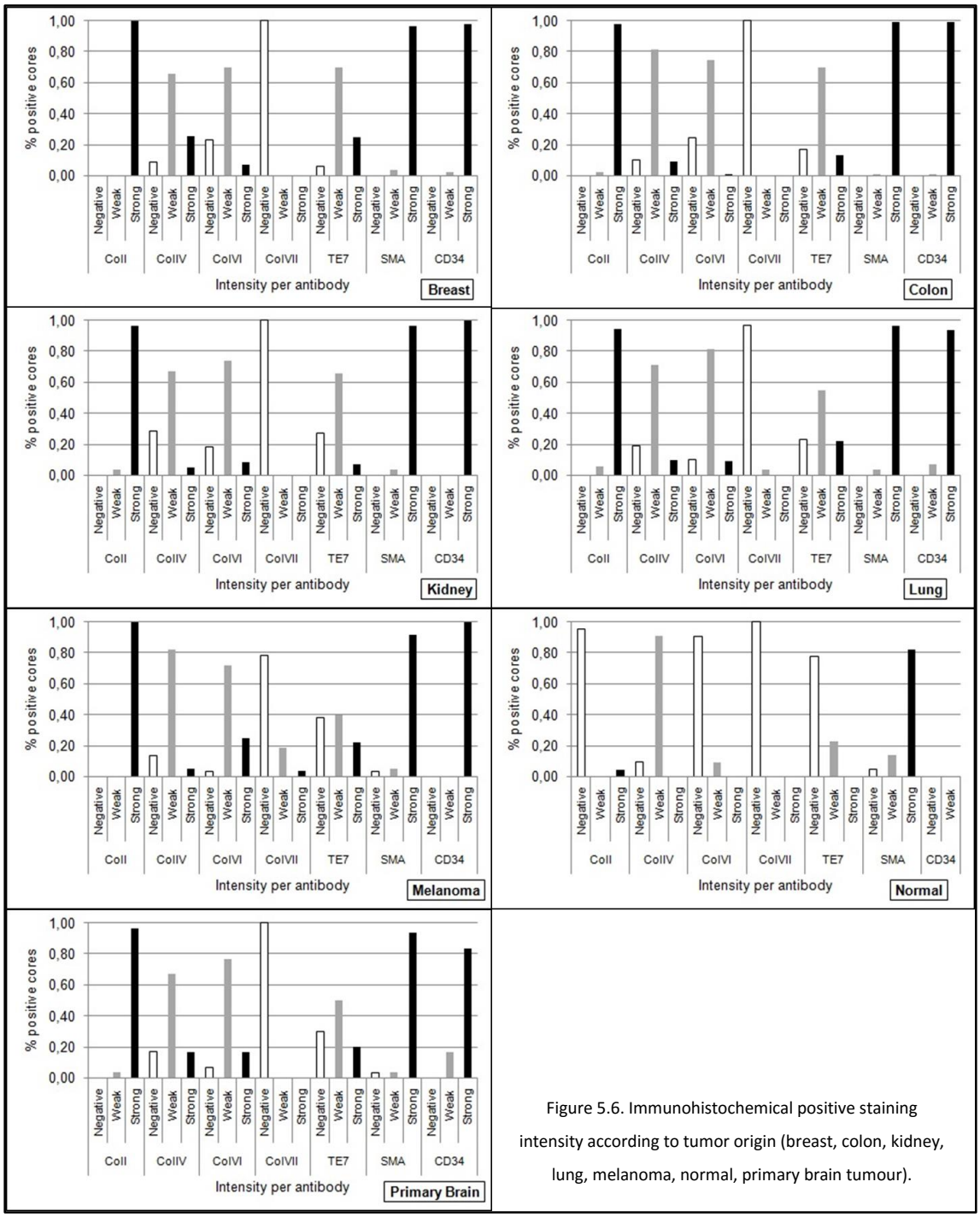

Figure 5.6. Immunohistochemical positive staining intensity according to tumor origin (breast, colon, kidney, lung, melanoma, normal, primary brain tumour).

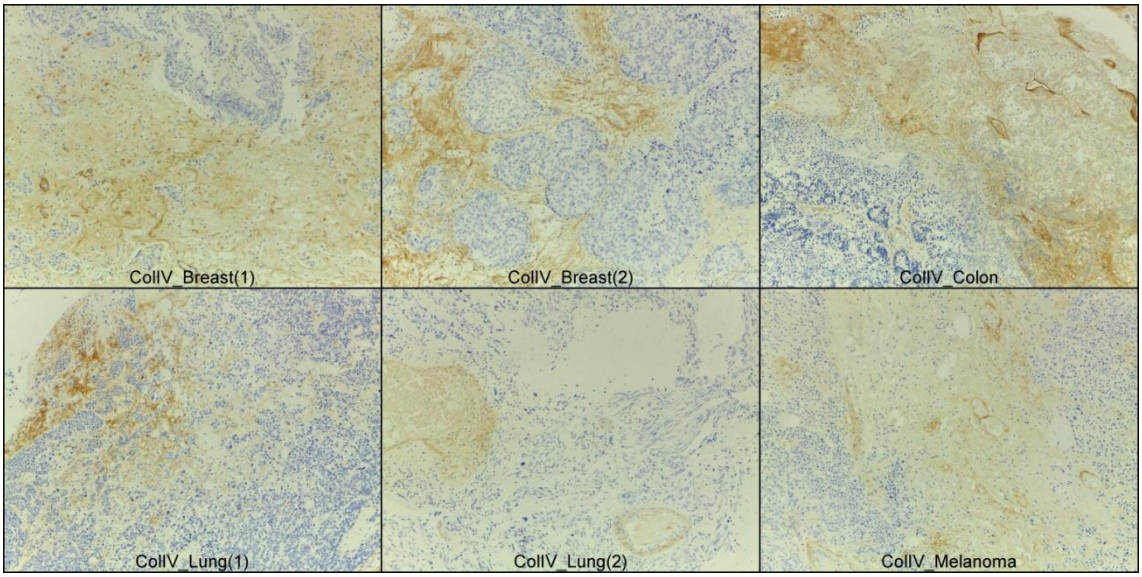

Figure 5.7. Examples of diffuse weakly stained areas using Collagen IV, on two samples of breast metastases, one of colon metastases, two of lung metastases and one of melanoma metastases.

Staining area of positive cores was measured using ImageJ (Figure 5.8 and 5.9). Normal control values were considered as a basal measure and were subtracted to the absolute data tumour cores obtained using the software (Figure 5.10).

Collagen I showed the most extensive immunoreactivity in all studied metastasis, followed by Collagens IV and VII, and anti-fibroblast TE-7. SMA and CD34, due to the structures they stain (smooth muscle and endothelial cells, respectively), had small staining areas.

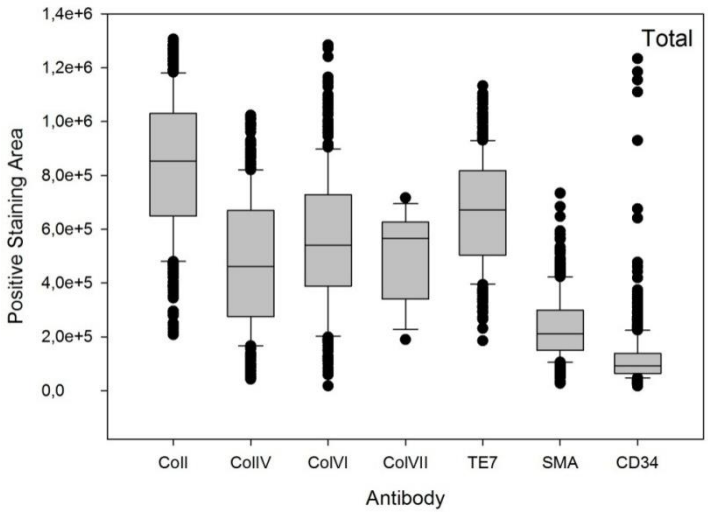

Figure 5.8. Positive staining area measured using ImageJ, per antibody.

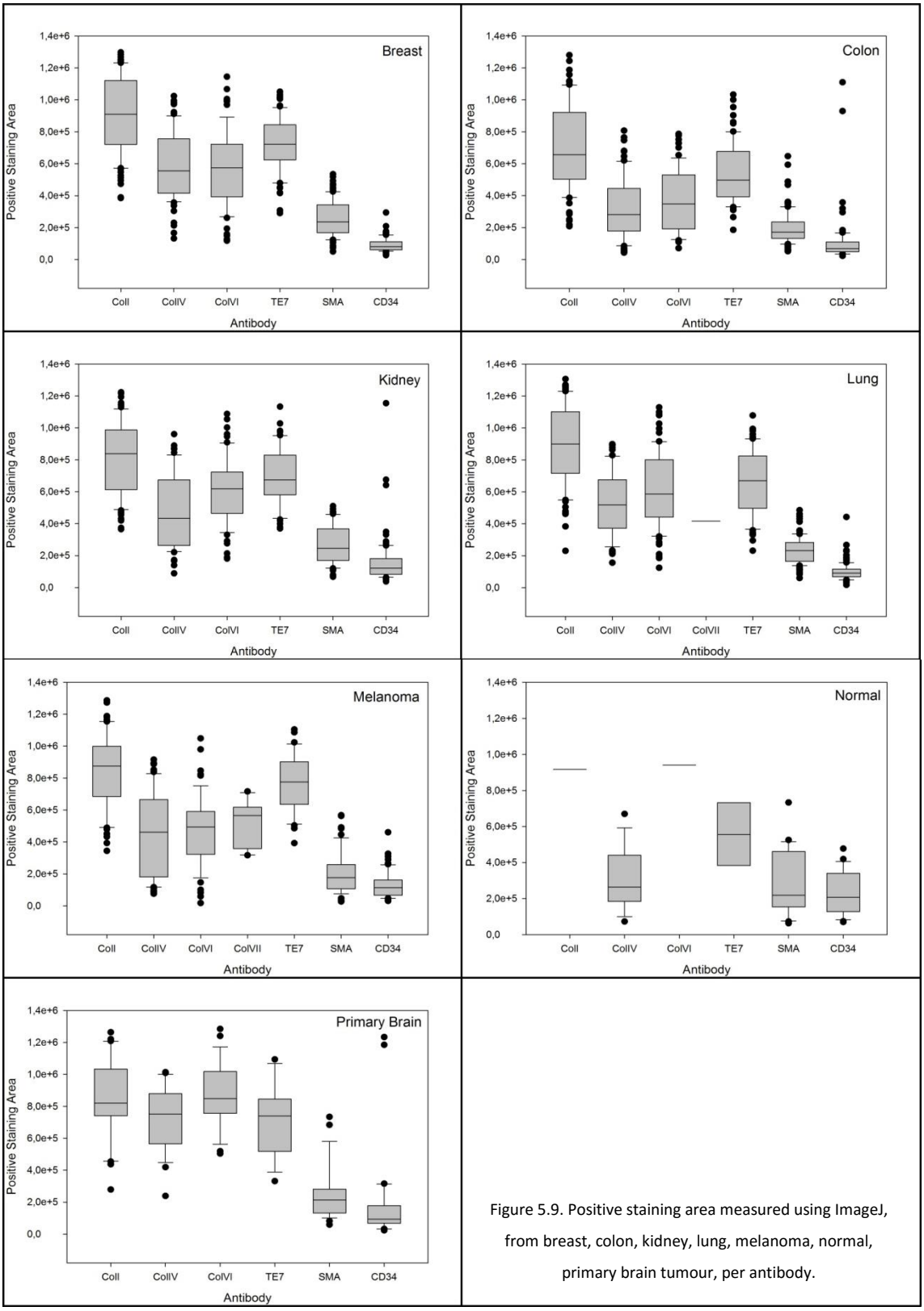

Figure 5.9. Positive staining area measured using ImageJ, from breast, colon, kidney, lung, melanoma, normal, primary brain tumour, per antibody.

In the collagen panel, Collagen I presented the most abundant reactivity in all brain metastases, while Collagen IV and VI showed similar results in the primary brain tumour control group (Figure 5.9).

On the other hand, when comparing with the obtained positive staining areas from the normal control samples, SMA and CD34 showed smaller positive staining area in all metastatic tumours as well as primary brain tumour control group, illustrated by negative bars in the figures bellow (Figures 5.10 and 5.11).

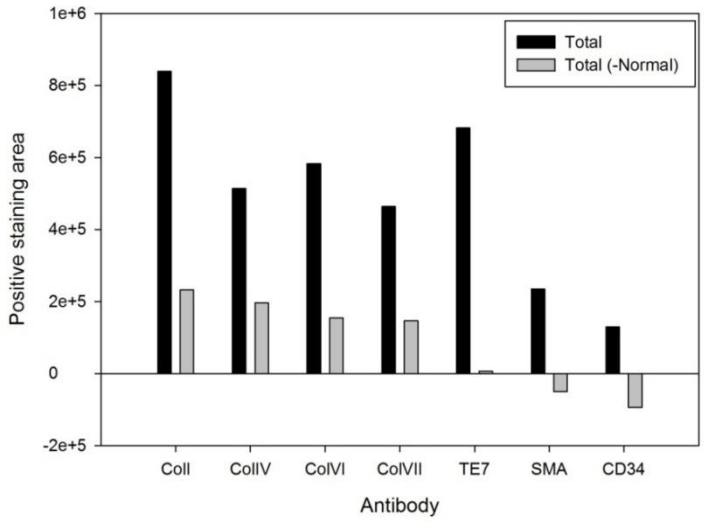

Figure 5.10. Positive staining area considering the normal control as a basal measure (defined as "(-Normal)"), using average values.

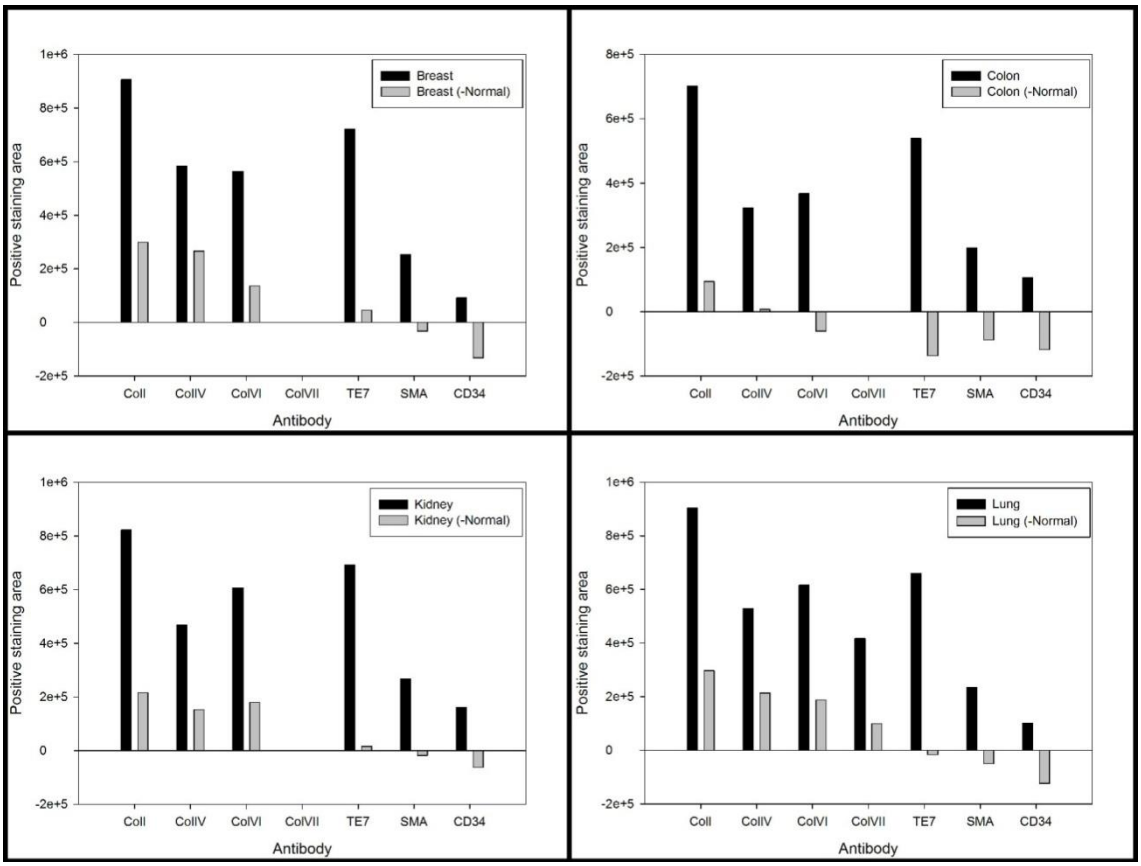

Figure5.11. Positive staining area considering the normal control as a basal measure (defined as "(-Normal)"), per tumour origin, using average values.

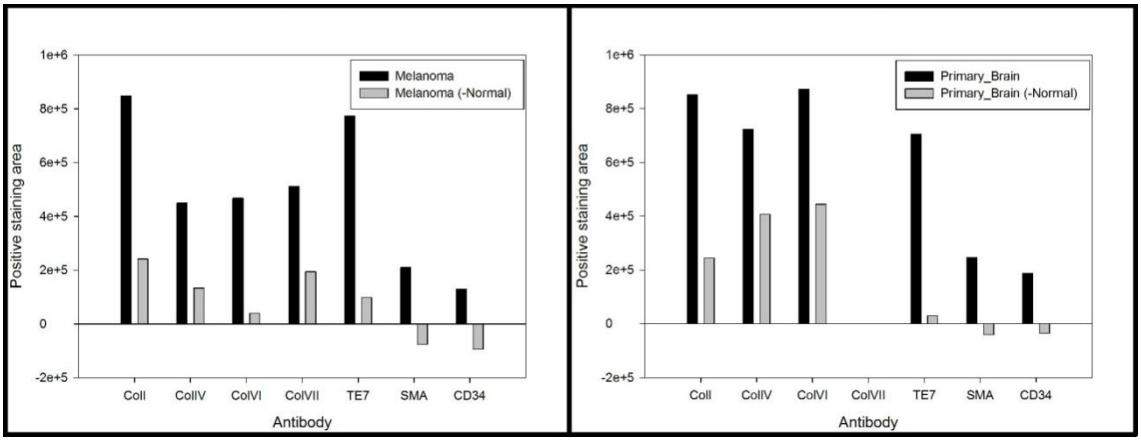

Figure5.11. (continued).

6. Discussion and conclusions

Several studies have already demonstrated the role of fibroblasts, or CAFs, in numerous cancers. Some examples include expression of pro-inflammatory factors in breast and ovarian cancer (71); EMT of prostate cancer cells (63); promotion of breast cancer cell adhesion to brain endothelial cells, invasive migration of breast cancer cells (73) and lung metastases (67); suppression of NK cells and malignant cell proliferation in hepatocellular carcinoma (62) and NK cell modulation in melanoma (73). Fibroblasts are found throughout the body, in the majority of tissues and organs. They are large spindle-shaped cells, with elongated nuclei, abundant endoplasmic reticulum and prominent Golgi, when activated (89). In physiologic conditions, these cells are responsible for the synthesis of various components of the ECM, like collagens, elastin, proteoglicans and other structural glicoproteins (89).

Despite the technical advances in pathology, like molecular biology, immunohistochemical detection of antigens remains the gold standard in tumour characterization for diagnosis, prognosis and therapeutic decision.
Fibroblasts, with mesenchymal embryonic origin, have a wide spectrum of phenotypic profiles, making them very difficult to distinctively identify and study (89), which constitutes a major limitation in research. As discussed above (see Introduction), the literature suggests various markers, in the absence of a specific biomarker. These include, besides vimentin and desmin, α-SMA, CD14, CD31, CD34, CD90, cellular fibronectin, hyaluronan, and several collagens (46–49).

In a preliminary phase, vimentin and desmin were included in the immunohistochemical panel of this study, as suggested in the reviewed researched papers. As expected, neither exhibited exclusive staining of fibroblasts. Vimentin has a limited value as diagnostic tool for the identification of fibroblasts, labelling a variety of other mesenchymal cells, including melanocytes, lymphocytes and endothelial cells, making it useful when used in combination with other markers (90). Desmin, staining an intermediate filament protein in striated and smooth muscle cells, leads to a very small number of positive tumours, like rhabdomiossarcomas, leiomyomas and

desmoplastic round small cell tumours (91). S100 protein was also included in the first immunohistochemical panel. It stains neuroectodermal components of brain, colon, kidney and melanoma, while lung carcinomas, breast carcinomas, colon adenocarcinomas and renal cells carcinomas are negative (92). Since obtained data from staining with these markers was scarce and unspecific, due to extensive staining of other tissue components on vimentin and most S100 protein slides, and lack of staining on desmin slides, the three were excluded from the study.

The panel of immunohistochemical markers chosen for the study was composed by Collagen I, Collagen IV, Collagen VI, Collagen VII, anti-fibroblast TE-7, α-SMA and CD34. Collagen I is the most abundant collagen in the human body and is putatively synthesized by fibroblasts (89). Besides, as described by several authors, tumours act as wounds that do not heal, which mimics a continuous wound healing reaction and where Collagen I plays an important role (69,89). It was expected that Collagen I, with the immunogen for the full length native protein corresponding to human collagen I (82), would be positive in the majority of metastatic samples, which was confirmed in this study.

Since fibroblasts produce, among other molecules, various types of collagens (89), the collagen panel was extended to Collagen IV, Collagen VI and Collagen VII.

Collagen IV is a major component of basement membranes, that separates parenchymal, endothelial and epithelial cells from underlying connective tissue(83). The used antibody stained 84.3% of the samples, corresponding mostly to blood vessels' basement membranes. Accordingly, capillaries were not stained. Yet, during microscopic observation, diffuse weakly stained areas were identified in some samples. These might correspond to areas of Collagen IV degradation or areas of unorganized and atypical basement membranes due to angiogenic processes. Some authors suggest that collagen IV degradation is important in metastization. Nakajima *et al*'s results (93) propose that highly metastatic breast adenocarcinoma tumour cells could degrade matrix significantly faster than poorly metastatic cells, through various mechanisms, some MMPs dependent and others independent, in lung metastases

(93,94). The obtained areas on our samples may correspond to Collagen IV degradation by tumour cells with a high metabolic rate, which are capable of invading and colonize brain parenchyma.

Collagens VI and VII are used routinely for myopathies (84,85). They were included in the studied collagen panel, since, as described before, collagens are produced by fibroblasts.

Collagen VI is a component of microfibrillar structures close to cells, nerves and blood vessels, widely distributed in several tissues, including brain, and forms a network between collagens I/III fibrils and basement membranes (84,95). This marker was positive in 80.6% of the studied samples, although mainly weak. Collagen VI is often deregulated in cancer (95), and besides structural support, it triggers signalling pathways involved in angiogenesis, via CD31, VEGFR1 and HIF1α (96), apoptosis (97), proliferation, fibrosis and inflammation (96,98). Adipose tissue is also a source of Collagen VI and obesity is a high risk factor for breast cancer (99,100). In breast cancer, in which Collagen VI was positive in 76.7% of samples, it has an anti-apoptotic effect, with primary tumour growth, whereas its depletion leads to diminished proliferation and increased tumour cell apoptosis. The staining that we observed in a large number of cases seems to confirm that a high expression of Collagen VI may be important for tumour growth and metastization, and it would be expected in brain metastases (96,100). In lung carcinoma, Collagen VI upregulates cancer cells motility (101), while its knockdown suppresses metastatic behaviour. In melanoma, Collagen VI ablation slows tumour growth, due to its role on tumor vasculature (102). Our finding of 90.0% and 96.7% positivity of lung and melanoma samples, respectively, were expected and may confirm the hypothesis that high collagen VI expression is important to tumour metastases.

Collagen VII was only positive in 3.4% of our samples, corresponding to a few lung and melanoma metastases. Wetzels *et al* (103) studied the distribution of this collagen in normal tissue and tumours, and concluded that its presence in tumours might be related to squamous differentiation and possibly not so important in tumoral origin. Probably for this reason, the obtained positivity for Collagen VII found in our study was

minimal. Our data, although interesting, are not likely to shed some light in collagen VII function in tumour metastases. Still, we observed staining in 3/90 of lung metastases cores and 13/60 of melanoma cores, and further studies investigating the differential expression in this tumour would be important.

Anti-fibroblast TE-7 has been exploited as a putative marker, even though no specific biomarker has been developed yet. Hayens *et al* (104) developed this antibody to specifically identify contaminating fibroblasts in thymic cultures. According to Goodpaster *et al* (47), whose study compares three different anti-fibroblasts markers, this antibody would be more specific than anti-fibroblast surface protein antibody 1B10 or anti-fibroblast growth factor receptor 4 antibody 5B5 antibodies either on human dermal fibroblasts cell culture or on normal human skin and human breast cancer FFPE samples. Pilling *et al* (46), aiming to distinguish between monocytes, macrophages, fibrocytes and fibroblasts, demonstrates that only fibroblasts, both in *in vitro* and human lung samples, express the combination of CD90, cellular fibronectin, hyaluronan and anti-fibroblast TE-7 markers. Yet, only 75.8% of our samples were positive, most of them weak. Anti-fibroblast TE-7 antibody seems to be a good marker, even though some differences were observed in our tumour samples, like 27.1% positivity for melanomas, and over 72.7% for other tumours. This may well reflect differential expression and could confirm the specificity of this antibody. The staining obtained with this marker is similar to the one obtained with Collagens I and IV. The use of this marker is, therefore, validated by our work, since it is helpful in the identification of fibroblasts in human brain metastases.

SMA stained, as expected, smooth muscle cells within blood vessel walls and apparent cells morphologically compatible with fibroblasts (49). Even if it was difficult to distinguish between each other, due to cross staining of differentiated smooth muscle cells, we consider this finding important, since SMA also stains myofibroblasts. With the exception of melanoma, with 96.7% positivity, all samples were mostly strongly positive, suggesting a high local concentration of SMA positive cells.

CD34, a cell surface endothelial cell marker, strongly stained the inner layer of blood vessels in all samples (88). As CAFs can arise from endothelial cells, through EndMT (54), this marker could be useful to determine CAFs origin. However, further analysis is needed, in order to unveil their origin.

As a general view, no specific immunostaining pattern could be observed among the studied samples. Cancer cells, except primary brain tumour cells and melanoma cells, were negative for all the used markers, which stained structures and cells located in tumour stroma (Figure 5.4). Consistent, overlapping staining of several antibodies was observed in stromal cells of our samples. The main difference was in staining intensity, where Collagen I, SMA and CD34 were mainly strong, and Collagen IV, Collagen VI and anti-fibroblast TE-7 were weak.

It is important to remember that “normal” brain samples belong to tumour samples, as distant from the tumour area as possible, given the fact of being difficult to obtain non-pathological brain that those not come from autopsy samples, which are prone to autolysis. This may explain the obtained positivities of Collagen I, Collagen VI and anti-fibroblast TE-7 in these samples, which should be deeper explored.

Nevertheless, as pathological structures and cells immunostaining is evaluated by comparing immunoreactivity obtained in normal ones, positive staining areas from normal control samples were subtracted to the areas from tumour samples.

Data showed that Collagen I, besides being the most abundant, has a bigger staining area than Collagens IV and VI in metastases samples. As normal samples displayed a small positivity area, whereas only one core stained, it is possible that Collagen I might be produced by cells that physiologically are not present in normal brain parenchyma, but rather in primary brain tumours or metastases, like CAFs (105).

The same reason, allied to normal samples origin, might explain the small staining areas of Collagen VI and anti-fibroblast TE-7 in colon and lung metastases samples. Indeed, anti-fibroblast TE-7 showed not only 22.7% positivity in normal samples, but also a large staining area, even bigger than the one measured in colon samples (that showed a comparatively smaller staining area staining area). These data support the hypothesis that this antibody should be used in brain samples to completely identify its

expression in brain tissue, in order to be correlated with fibroblast staining. To our knowledge, there are no published references using TE-7 in brain tissue, and our finding is original, and should be pursued further. Additionally, as described above, melanoma samples had only 27.1% positivity for anti-fibroblast TE-7, and most metastases samples had a weak immunostaining, which can be not a disadvantage, but rather an indicator of good specificity to fibroblasts staining.

Also with a comparatively smaller staining area, when comparing absolute areas with the normal control group, were SMA and CD34. These two markers stained specifically cells in blood vessels, one of the possible origins of CAFs. As their positivity was high in the studied samples, the smaller areas measured could be linked to a defective angiogenesis or even with CAFs origin (7).

Glioblastomas are very heterogeneous and complex tumours (79), with regions of necrosis and haemorrhage, and microscopic areas of pseudopalisading necrosis, pleomorphic nuclei and microvascular proliferations. Genetically, glioblastomas have several deletions, amplifications, point mutations and subclones of tumour cells, which contribute to its aggressiveness, complexity and poor outcome (106). As these tumours have a high rate of angiogenesis (107), Collagen IV, SMA and CD34 positivity in blood vessels walls and endothelial cells was expected, and completely correlated with our findings. Yet, the remaining immunostainings were interesting. Glioblastomas were positive for Collagen I, Collagen VI and anti-fibroblast TE-7. Tumour cells can synthesize Collagens I and VI and stromal cells, while normal brain parenchyma surrounding the tumour, can produce Collagen IV (79,108,109). According to Mammoto *et al* (109), tumour stroma controlled by collagens is important for angiogenesis and tumour progression. These collagens have three important roles in brain tumours, namely sites of adhesion, reservoir of molecules and activation of networks for tumour growth, differentiation and invasion (79). Our results proved the presence of TE-7 positive CAFs-like cells in glioblastomas, which is a novel finding *in vivo* samples, although it has been described in glioma cells *in vitro* (105). Although there are a few studies on this topic, further analysis is needed in the context of collagens and Anti-fibroblast TE-7 expressions in glioblastoma, to evaluate its role in disease progression and tumour recurrence.

6.1. Limitations

The study faced several limitations. The biggest was the absence of a specific biomarker for fibroblasts, which, nevertheless, was partially overcome by using an extended immunohistochemical panel, where brown staining in tumour stroma was considered positive. The choice of markers was also limited to the ones available in the laboratory where the study took place, with the exception of Collagens I, IV and anti-fibroblast TE-7 (46,47).

Sample number was limited by the cases existing in the laboratory archive where the study was performed. Due to inclusion criteria previously defined, it was not possible to collect thirty kidney and melanoma metastases, as it was done for breast, colon and lung samples.

Normal control samples were also limited, because they belong to tumour brain samples, instead of non-pathological brain. Hence, we could not be entirely sure they would be completely “normal”.

6.2. Suggestions for further studies

In the context of the identification of CAFs in brain metastases, further studies are needed. A larger immunohistochemical panel, to include markers like FSP1 (48,49), might strengthen the obtained data in this project.

Several research groups are investigating different molecules to specifically identify fibroblasts, like Snail1, a transcriptional factor (110), whose expression has been associated with CAFs, for example, in colon cancer (110). Its upregulation enhances cancer cell migration, whether its depletion causes a significant decrease in it (110).

Other metastatic tumours, like prostate metastases, should also be studied. Counting cells morphologically compatible with fibroblasts can also be performed, to try to relate it with the immunostaining data obtained previously.

Assuming that cancer progresses from the primary tumour to extra-cranial metastases and brain metastases, the study of these three stages may reveal important data on CAFs origin in brain metastasis.

6.3. Conclusions

Our data provide compelling evidence for the presence of CAFs in most frequent brain metastases and are similar to previous published ones, like Duda *et al* (49), who detected benign-appearing SMA positive cells with typical fibroblast appearance in the extravascular areas of the majority of their brain metastatic cases. The immunohistochemical markers used in our study (Collagens I, IV, VI and VII, anti-fibroblast TE-7, SMA and CD34), on brain metastasis from breast, colon, kidney, lung and melanoma, was able to identify cells and structures compatible with the presence of CAFs in them.

Nevertheless, the identification of CAFs in brain metastases not only increases oncobiological knowledge, but also may reveal a potential novel therapeutic target.

As reviewed above, stroma has an important role in brain metastases and actual therapeutic strategies for managing this disease, namely whole brain radiotherapy, stereotactic surgery, surgical resection and chemotherapy, are very aggressive and with limited effectiveness (43). Stroma-targeted therapies are being exploited and becoming more popular in primary tumours and extra-cranial metastasis (30–33). As CAFs are important in tumour progression, its identification can bring new and useful strategies in brain metastases management.

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Appendix I. Detailed Hematoxylin-Eosin Protocol

The following protocols are used currently in the *Laboratório de Neuropatologia*, *Hospital Santa Maria*, *Centro Hospitalar de Lisboa Norte*.

Reagents

- 99,9%, 96% and 70% **Alcohol** (Proclinica or Aga);
- 25% **Ammonia** for analysis (Panreac, Ref. 121129.1611);
- **Chloridric Acid** (Pronolab, Ref. CAS 7647.01.0);
- **Distilled water**;
- **Eosin Yellowish** for microscopy and indicator (Merck, ref. 6357863);
- Papanicolau's Solution Losung 1a **Harris Hematoxylin Solution** for cytological cancer and cycle diagnosis (Merck, Ref. 1.09253.0500);
- **Xylene** (Aga, Index 601-022-00-9).

Solutions

- **Differentiator: 1% Chloridric Alcohol**

Chloridric acid	1mL
70% Alcohol	99mL

- **Blueing solution: 1% Ammoniacal Water**

25% Ammonia	1mL
Distilled water	99mL

- **Eosin Working solution: 1% Alcoholic Eosin**

Eosin	1mL
70% Alcohol	99mL

Procedure

1. Dewax sections in xylene for 15 minutes;
2. Rehydrate in decreased concentrations of alcohol to tap water;
3. Incubate in **Hematoxylin** for 4 minutes;
4. Rinse in tap water;
5. Immerse sections in the **differentiator** for 2 seconds;
6. Rinse thoroughly in tap water;
7. Immerse sections in **blueing solution** for a few seconds, until they turn bluish;
8. Rinse in tap water;
9. Incubate slides in **eosin working solution** for 10 minutes;
10. Dehydrate rapidly in increased concentrations of alcohol;

11. Clarify in xylene;
12. Mount with synthetic mounting media and coverslips.

Results

- **Nuclei** – Purple to Blue;
- **Cytoplasm** – Pink shading.

Appendix II. Detailed Immunohistochemical Staining Protocol

The following protocols are used currently in the *Laboratório de Neuropatologia*, Hospital Santa Maria, Centro Hospitalar de Lisboa Norte, EPE.

Reagents

- 99,9%, 96% and 70% **Alcohol** (Proclinica or Aga);
- 25% **Ammonia** for analysis (Panreac, Ref. 121129.1611);
- **Antibodies** (see table 4.1. above);
- **Antigen Unmasking Solution** Low pH (Vector, Ref. H-3300);
- **Chloridric Acid** (Pronolab, Ref. CAS 7647.01.0);
- **Dako REAL™ Envision™ HRP Rabbit/Mouse**, Ready-to-use (Dako, Ref. K4007);
- **Dako REAL™ Kit Substrate Buffer and DAB$^+$ Chromogen** (Dako, Ref. K5007);
- **Distilled water**;
- **30% Hydrogen Peroxide** (Merck, Ref. 1.07210.0250);
- **Novocastra™ Novolink™ DAB** (Polymer) (Leica, Ref. RE7230-K);
- **Novocastra™ Novolink™ Polymer Detection System** (Leica, Ref. RE140-K);
- Papanicolau's Solution Losung 1a **Harris Hematoxylin Solution** for cytological cancer and cycle diagnosis (Merck, Ref. 1.09253.0500);
- PBS Buffer solution;
- **Tris-EDTA solution**, 0,5mM in water (Sigma-Alderich);
- **Triton X-100** (Sigma-Alderich);
- **Xylene** (Aga, Index 601-022-00-9).

Solutions

- **Phosphate buffered saline (PBS)**
- **Hydrogen Peroxide Working solution**

30% Hydrogen Peroxide	6mL
Distilled water	250mL

- **Antigen Retrieval Working solution**

Antigen Unmasking Solution Low pH	9,375mL
Distilled water	1000mL

- **Antigen Retrieval Working solution for TE-7 antibody**

Tris-EDTA	2mL
Triton X-100	0,25mL
Distilled water	1000mL

- **DAB Revelation Solution** (both Dako and Novocastra)

DAB$^+$ Chromogen	20µL
Substrate Buffer	1mL

- **Differentiator: 1% Chloridric Alcohol**

Chloridric acid	1mL
70% Alcohol	99mL

- **Blueing solution: 1% Ammoniacal Water**

Ammonia 25%	1mL
Distilled water	99mL

Procedure

1. Dewax sections in xylene for 15 minutes;
2. Rehydrate in decreased concentrations of alcohol to tap water;
3. Incubate in **Hydrogen Peroxide Working solution** for 20-25 minutes at RT;
4. Rinse in distilled water;
5. Immerse slides in **Antigen Retrieval Working solution or Antigen Retrieval Working solution for TE-7 antibody** and put in the microwave for 10 minutes at 600watts, followed by another 20 minutes at 850watts;
6. Let the solution cool down and wash with PBS;
7. Incubate sections with a **primary antibody**, according to the conditions specified before (see Table 4.2);
8. Rinse thoroughly with PBS;
9. Apply the **Novocastra™ Novolink™ Polymer Detection System** (for the anti-Collagen I and anti-Fibroblast antibodies) or **Dako REAL™ Envision™ HRP Rabbit/Mouse** for 2 hours at room temperature;
10. Rinse sections with PBS;
11. Stain with the DAB Revelation Solution for 5 minutes;
12. Rinse thoroughly with tap water;
13. Counterstain with Hematoxylin (see steps 3 to 9 in Hematoxylin-Eosin Staining Protocol in the previous appendix);
14. Dehydrate rapidly in increased concentrations of alcohol;
15. Clarify in xylene;
16. Mount with synthetic mounting media and coverslips.

Results

- **Positive structures** – Brown;
- **Background** (nuclei) – Blue.